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Aquaculture Feed Grains Program

Incorporating:

GRDC Project UWA00062: Development of value-added plant protein products for the aquaculture feeds sector

and

FRDC Project 2004-236: Aquaculture Nutrition Subprogram - Evaluation of Value-added Grain Protein Products for Atlantic Salmon and Black Tiger Prawns

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Non Technical Summary

Aquaculture Feed Grains Program

GRDC – UWA00062 Development of value-added plant protein products for the aquaculture feeds sector

FRDC – 2004-236 Evaluation of Value-added Grain Protein Products for Atlantic Salmon and Black Tiger Prawns

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Objectives

1. Development of value-added lupin protein product(s) for use in the animal feeds sector.
2. Evaluation of the nutritional value of a range of value-added lupin protein products when fed to fish.
3. Commercial transfer of intellectual property for development of new-product(s).
4. To determine the nutritional value of selected grain products developed as part of the linked CLIMA-GRDC project, when included in feeds for Black tiger prawns and Atlantic salmon.
5. To evaluate any potential nutritional limitations of the grain products in aquaculture feeds.
6. To provide grain producers, grain processors, aquaculture feed manufacturers and the prawn and salmon aquaculture industries with information about the nutritional characteristics and quality assurance criteria of grain products so that they can be marketed and used with confidence in aquaculture feed formulations.

This program represents a major collaborative initiative between the Grains and Fisheries Research and Development Corporations. It has engaged seven different research providers and three industrial collaborators in achieving its outcomes. Numerous findings were encountered through this program, which are collated in this report. Key among those findings is:

- The dehulling of lupins significantly improves their nutritional value to fish. A linear increase in digestible energy value was observed, while a curvilinear response in digestible protein value was observed. This finding shows that there is signfiicant nutritional benefit to the fish in optimising the dehulling efficiency of lupins, but in terms of protein value that a minor contamination with hulls is unlikely to significantly reduce the protein value.
- Considerable variability in the digestible protein and energy value of the lupin kernel meals was observed. It was shown that this variability could be assessed as a function of grain composition. Higher protein levels in the meal correlated with better protein and energy digestibility. The high protein levels also correlated with lower non-starch polysaccharide (NSP) levels in the kernel meals. This resulted in a concommitant relationship between protein and NSP and digestibility. Assessment of the fibre composition of the kernel meals also showed that lignin was a key fibre class that affected protein digestibility, with higher

lignin levels strongly correlating with poorer protein digestibility.

- Considerable variability in the composition of lupin kernel meals was observed among the 76 samples evaluated for digestibility. As protein increased in each lupin kernel meal a reciprocal decrease in NSP was observed. Across three years worth of sample collection of commercial cultivars from a single site, significant variation in composition was observed. Variation in composition was greater across years than across cultivars.
- The use of near-infrared spectroscopy (NIRS) was shown to be able to provide rapid and useful assessments of not only crude composition of whole grain and kernel meals, but also their digestible protein and energy value. This should allow grain processors and users to rapidly and more accurately assess the actual value of discrete batches of grain products.
- The alkaloid gramine was shown to be a significant anti-nutritional factor to rainbow trout. When included in diets at levels above 100 mg/kg there was a dramtic decline in feed intake and subsequently growth of the fish. No other pathological issues were identified with the inclusion of gramine in the diet and its main mode of anti-nutritional activity is through reducing palability to the animal. This explains why certain genotypes of *Lupinus luteus* (yellow lupin) are not well tolerated and utilised in fish diets and provides clear guidelines for plant breeders as to what critical target alkaloid levels need to be.
- Two levels (15% and 30%) of lupin kernel meals were included into diets of rainbow trout and used to demonstrate that protein and energy utilisation is not deteriorated by their inclusion. This finding provides support for the notion that the plant protein and energy content is as effectively utilised as animal protein by carnivorous fish in their diet.
- The extent of the influence that the variability in the digestible protein content of lupin kernel meals had on fish growth was assessed in two separate experiments. The first experiment used low-protein diets (350 g/kg) and high-inclusion levels (40%) of a low digestibility and high digestibility lupin kernel meals. These diets were then fed at a range of ration levels from starvation to satiety to examine both palatability and utilisation aspects of the feeds. The results demonstrated that a significant effect of the lower digestibility lupin kernel meal could be measured as an effect on growth using this design. A second experiment examined the effect of the same raw materials at more conservative inclusion levels (25%), in diets formulated to more typical commercial specifications (400 g/kg protein, 250 g/kg lipid). In this second experiment the variability in digestible value became masked, demonstrating that under commercial equivalent conditions that variability in digestibility of lupin kernel meals would be unlikely to be observed.
- Using both protein concentration and isolation techniques, a series of protein concentrates and isolates were prepared from *L. angustifolius*, *L. luteus* and *L. mutabilis* kernel meals. Using protein isolation methods it was possible to produce products with protein levels in excess of 80%. Protein concentration methods produced products of a lower protein content, but had a greter yield. Both yield and and protein content will be important factors in determining the commercial viability of the final products.
- Different drying methods were examined in the production of protein isolates because of their importance in cost of product manufacture and also their influence on product quality. Freeze-drying proved to be a useful experimental/laboratory scale method, but it was not considered a viable industrial scale method. Up-scaling the processes involved examining spray-drying and ring-drying technologies. Both *L. angustifolius* and *L. luteus* protein isolates were examined in each drying process. Spray-drying proved to produce good consistent product, while ring-drying caused the product to gum and not produce a useful product.

- Digestibility evaluation of the prototype LPC's showed that they had highly digestible protein and energy characteristics, irrespective of lupin variety used to produce the product. These digestibility parameters were assessed using both internationally used faecal collection methods of settlement and stripping. A comparison of the results obtained using either methods showed that stripping gave more conservative estimates and that the disparity between the results was greater when the test diets had greater levels of carbohydrate material.
- Inclusion of the prototype LPC's in feeds for rainbow trout was shown to not hinder their growth or feed intake. It was also demonstrated that, provided the dietary amino acids were balanced, then the fish used the LPC's as effectively as they used fish meal protein.
- Different drying processes were observed to affect the composition of LPC's, with use of high-temperature drying resulting in lower protein and higher fibre levels. Although these drying effects did not deteriorate the digestible value of the LPC's an assessment on their nutritional values showed that although the fish could digest them well, they were not used as efficiently for growth and therefore had reduced value as a feed material. Similar such deterioration of the LPC's was not observed when the product was dried using spray-drying technology.
- An improved reactive lysine assay was developed to assess nutritional damage caused by the high-temperature drying of the LPC's. This assay effectively measured the proportion of lysine within a sample that had its tertiary amino group unavailable chemically. It was shown that the high-temperature drying of the LPC's resulted in an increased level of unreactive lysine most likely due to chemical condensation of a carbohydrate molecule to this tertiary amino group. This means that the lysine becomes unavailable for use in protein synthesis, supporting the observations from the fish growth study.
- Comparison of the digestibility responses between rainbow trout and Atlantic salmon showed that there was a high-degree of homology between the two species in respect to their response to different grain products. Although the actual digestibiity values obtained for the same products differed between each species, the relative responses were similar. This supports that either species provides a useful indication of the likely response of the other to digestibility of feed grain products.
- Five different varieties of *L. angustifolius* kernel meal were examined for their variability in digestibility parameters when fed to Atlantic salmon. Significant variability was observed in crude protein digestibilities from each of the kernel meals. Ingredient protein digestibility in the Atlantic salmon ranged from 66.1% to 94.8%.
- The influence of lupin kernel meals, soybean meal and a lupin protein concentrate on gut transit in Atlantic salmon was examined using a marker replacement method. The results of this work showed that the inclusion of lupin kernel meals increased the rate of gut transit of the feed compared to the effects induced by the inclusion of soybean meal or a lupin protein concentrate.
- The inclusion of lupin kernel and soybean meal in diets for sea-water reared Atlantic salmon was examined at two inclusion levels and at two water temperatures to examine if there was any influence of diet raw material on temperature response. Feed intake and growth response was improved from fish fed the lupin kernel meal diets compared to both the fish meal based reference and the soybean meal diets. This improved performance of the lupin kernel meal diets was observed at both water temperatures. No interaction effect of temperature and ingredient was observed in the study. These findings show that lupin kernel meals have a significant advantage over soybean meal when included in diets for sea-water reared Atlantic salmon.

- The inclusion of lupin kernel meals (*L. angustifolius* and *L. luteus*) and protein isolates were shown to not have an effect on intestinal enteritis in Atlantic salmon, contrary to the effect observed when soybean meal is included in their diet. This anti-nutritional activity of soybean has been shown to be a negative feature of this grain product and is not shared by lupin products. The inclusion of lupin kernel meals in diets for Atlantic salmon was also shown to positively influence the lipid digestion from the diet, whereas soybean meal did not.
- Eight commercially supplied products, from two grain processing companies, were evaluated in a series of commercial-in-confidence studies in rainbow trout. In addition to the product assessment studies, samples from each of the lupin kernel meals studied for the NIRS assessment were also provided, along with the accompanying data, to the three project commercial partners for their own development of NIRS calibrations.
- Significant variability in the digestibility of protein and energy was observed from 12 different samples of lupin kernel meals fed to prawns. Digestibility of protein ranged from 92.7% to 96.8% and digestibility of energy ranged from 69.6% to 77.2%.
- Growth of diets containing up to 50% of the diet as lupin kernel meal showed that prawns used this raw material as effectively as fish meal and also soybean meal. No decline in feed intake was observed even at the highest inclusion levels, supporting that commercial application of lupin kernel meals to prawn diets is unlikely to negatively affect growth or feed intake.
- The inclusion of dietary alkaloids in feeds for prawns was shown to have some impact on feed intake, but was not as clear as the response observed from fish. The alkaloid gramine when included in prawn diets was observed to leach from the diets after being fed to the prawns and this affected the assessment to a degree.
- From common lupin kernel meals studied in rainbow trout, prawns and Atlantic salmon a comparison of the digestibility of protein and energy was made among the three species. No significant relationships were observed among any of the species. It is suggested that because of low levels of variability in the digestibility values of the tested lupin kernel meals, it was difficult to define possible inter-relationships in these parameters among the species. Differences in experimental methods and laboratory routines also make direct comparison difficult.
- Lupin kernel meal inclusion in an extruded pellet was examined at 0%, 10%, 20% and 30% inclusion levels. An increase in pellet hardness, bulk density and sink rate was observed with increasing lupin inclusion. The relationship was generally curvilinear, with maximal responses occuring at around 20% inclusion. Extruded pellet expansion and vacuum oil uptake were generally reduced with increasing lupin inclusion. Water retention in the extrusion mash was also enhanced by the inclusion of increasing levels of *L. angustifolius*, *L. luteus* or soybean meal.
- Significant variability in diet extrusion features was observed as a function of different lupin varieties/culitvars and also the actual species of feed grain being included in a diet. The inclusion of lupin kernel meals (from either *L. angustifolius* or *L. luteus*) was shown to increase bulk density, sink rate and pellet hardness and decrease vacuum oil uptake and pellet expansion, at a different degree than that achieved by a similar inclusion of soybean meal. However, the degree to which each factor was affected varied depending on grain product and its inclusion level.

- Extrusion of fish diets significantly improves their digestible energy value, but has limited effect on the digestibility of other diet parameters. However, extrapolation of diet digestibility parameters to examine ingredient digestibilities shows that there is limited correlation between extruded and non-extruded diets in terms of their protein digestibility, but that energy digestibilities remain highly correlated.
- Numerous publications and media have arisen from this project.
- Aquaculture feed industry partners have begun adoption of the use of lupin kernel meals in their products.
- Grain processing industry partners have initiated the large-scale commercial dehulling of lupins for the domestic and international aquaculture feed markets.

1.0 Introduction

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1.1 Pursuing greater value for grains

The profit a farmer yields from grain production in its simplest format is a combination of; the costs of production *x* yield per unit area *x* value of grain per tonne. In attempting to improve profitability of grain production significant work has gone into improving agronomic practices to reduce the cost of production and improving genetic traits to improve yields. However, addressing issues that affect value have been somewhat more difficult. The difficulty in addressing value criteria, for a grain like lupins for example lies in that the value is affected by a large array of factors, many of which are independent of factors at the control of the farmer or scientist. For example, exchange rates, volume of competitor products, trade tariffs etc all impinge on the potential value of the grain in any given market.

Although many facets of the grain value are difficult to control, certain elements can be managed to optimise potential value. For example, the value of protein grains like lupins is largely benchmarked against the international soybean meal price on a protein parity basis. This means that a 30% protein lupin is generally valued at 62% the value of a 48% protein soybean meal, while a 38% protein lupin kernel meal is generally valued at 80% the value of a 48% protein soybean meal. Because of this relationship, any gains in the overall protein content of the grain drive the relative value of the grain higher in the international protein trade market.

Another mechanism of grain value enhancement is grain quality segregation. In the Australian wheat industry over 12 different segregations exist for different wheat classes depending on wheat variety, protein level and other quality features. For different wheat varieties/classes higher values have been obtained as a consequence of this segregation. For a feed grain however, the key price-determining attribute is its digestible protein and/or energy value. Therefore by identifying higher grades according to these criteria it may be possible to produce different grades of feed grain that accordingly will have greater market value.

A third mechanism of grain value enhancement is identification of point-of-difference features or functional properties. Certain raw materials are used in some products not because of their nutritional values, but because of properties they bring to the product through their specific functional properties. An example of this is wheat gluten, which has useful binding properties as a protein, and accordingly its value is considerably higher than what would be achieved on a simple protein-parity basis. Other point-of-difference features include the removal of anti-nutritional factors (ANF) from some protein meals. Bioprocessed soybean meal, like HP340 (Hamlet Protein, Horsens, Denmark), has a significantly lower level of most of the ANF present in soybean meal (Refstie et al., 1998). Because of this reduction in ANF the HP340 has a significantly higher value in the international feed market and is widely used in pet foods, calf-milk replacers and aquaculture feeds.

Although there are several mechanisms for increasing the value of a grain, the potential for this needs some temperance as it is still largely influenced by other factors. The key value to

understanding many of these quality features of grains is therefore not to just to seek to gain greater value, but to maintain capacity to market specific grains in an increasingly complex and demanding market place and thereby maintain market presence. For if quality criteria are eroded away at the expense of other traits then gains made in one area can be just as quickly lost through a reduction in relative market value and complete loss of key markets.

1.2 Addressing feed resource risk in the aquaculture sector

Aquaculture is recognised as one of the fastest growing animal production industries in the world, particularly so in the Asian region (Tacon, 2004; Lungren et al., 2006). However, the identification and development of alternative protein resources to the use of fish meal in aquaculture diets remains a high priority for improving the sustainability of aquaculture and reducing feed formulation risk. Fishmeal has traditionally been considered an important protein source for use in aquaculture diets for both carnivorous and omnivorous species, and many aquaculture formulations still have fish meal included at levels in excess of 50%. However, being too reliant on any one ingredient presents considerable risk associated with supply, price and quality fluctuations. As a strategy to reduce risk, the identification, development and use of alternatives to fish meal in aquaculture diets is a high priority. Due to the volumes of fish meal and oil used in aquaculture, especially for carnivorous species, aquaculture of these species is still perceived as a net fish consumer rather than producer and this practice has raised concerns about the long-term sustainability of these industries (Naylor et al., 2000).

To improve resource security and reliability for aquaculture feeds, one option has been to increase the use of alternative meals and oils as feed ingredients in diets for aquaculture species (Glencross et al., 2007). Indeed, substantial effort has been expended over the past decades in evaluating a wide range of potential alternatives to fish meals and fish oils for use in aquaculture diets. Those ingredients can generally be classified into those being derived from either plant origin or terrestrial animal origin. Plant derived resources include: soybean meals, protein concentrates and oils (Kaushik et al., 1995; Refstie et al., 1998; 1999), canola meals, protein concentrates and oils (Higgs et al., 1982; Mwachireya et al., 1999; Forster et al., 1999; Burel et al., 2000; Glencross et al., 2004a) and lupin meals and protein concentrates (Burel et al., 2000; Farhangi and Carter, 2001; Booth et al., 2001; Glencross et al., 2003a; 2004b; 2004c). Key potential terrestrial animal ingredients have included resources such as rendered meat meals (Bureau et al., 1999; 2000; Stone et al., 2000; Sugiura et al., 2000; Williams et al., 2003), blood meals (Bureau et al., 1999; Allan et al., 1999) and poultry meals (Bureau et al., 1999; Nengas et al., 1999). However, the application of alternative ingredients/raw materials depends on the type of diet to which the ingredient is being applied.

Typically aquaculture diets fall into one of three spectrums; (1) high-nutrient-density diets, which are high protein, high fat diets made for fish such as Atlantic salmon, rainbow trout, barramundi and yellowtail kingfish, (2) low-nutrient-density diets, which are low protein, low fat diets made for fish such as catfish, tilapia and carps and (3) crustacean-diets which are moderate protein, low fat diets made for species such as tiger prawns, but the diets have other constraints such as a need for high levels of attractants and extended water stability features. The value of the diets, and with that their purchasing leverage in paying premiums for premium ingredients, is directly related to the protein and energy content of the diets – the higher the protein and energy, the greater the potential purchasing leverage.

1.3 Feeding plant protein meals to fish

Feed grains have considerable potential to supply dietary nutrients and energy for fish. These resources have generally been shown to provide promising levels of digestible and available nutrients and energy. However, the optimisation of the use of these raw materials in aquaculture diets requires a detailed understanding of their chemical composition and the consequences of feeding these materials and their influence on each specific species being fed.

The use of feed grains in fish diets can also introduce a suite of problems. Not only does the use of high-levels of plant proteins increase the potential for inducing essential amino acid limitations, many plant derived feed resources also contain a variety of anti-nutritional (biologically active) factors (ANF). The influence of these ANF on fish can be considerable, varied and is not well understood (Francis et al., 2001).

In assessing the value and potential of a range of feed grains there has been considerable research on the use of feed grain resources in the diets of a variety of aquaculture species (Gomes et al., 1995; Booth et al., 2001; Glencross et al., 2007). However, despite this, there still remains need for targeted research on identifying key attributes and limitations to the use of particular feed grains in aquaculture diets to encourage industry to more confidently adopt their use. This is particularly the case with those feed grains that have been identified as having potential, but which do not have a lot of sound data on their application in diets of particular target species.

Soybean meal is one feed grain resource that has been widely used in aquaculture diet formulations with considerable success and there is a large amount of data underpinning the acceptance of this raw material (Kaushik et al., 1995; Refstie et al., 1998, 1999). However, in Australia there is limited production of soybeans, but substantial production of lupins, canola and field peas. Each of these grains has been shown to provide some value as a potential aquaculture feed ingredient (Gomes et al., 1995; Burel et al., 2000; Booth et al., 2001).

1.4 Developing the application of grain protein products for the aquaculture sector

Considerable effort has been focused on the extension and development of feed grains for the aquaculture sector since the late 1980's internationally and early 1990's in Australia. Of those feed grains evaluated, lupins have consistently emerged as one of the most viable options for use in modern nutrient-dense aquaculture diets (Glencross, 2001). Because of this there has been a continued concerted effort to promote lupins as an aquaculture suitable feed ingredient for both domestic and export use. There now exists within the international literature, considerable information on the value of lupin meals for a range of different aquaculture species (Burel et al., 1998; Faranghi and Carter, 2001; Glencross and Hawkins, 2004; Glencross et al., 2005; 2006; 2007).

One limitation identified in the data set is how the aquaculture feed manufacturers perceive lupins and the availability of information on their use. Most modern aquaculture feed manufacturers now formulate diets based on the level of nutrients available (digestible) to the target species. To achieve this the formulator requires data on the digestible value of the ingredients to be used so as to allow linear least-cost formulations to be achieved. Therefore the determination of digestible value data is becoming increasingly important.

Another of the clear deficiencies in the knowledge of lupin meal use in aqua-feeds is its unknown level of nutritional variability. While key assessment criteria of the meals are usually the protein, fibre and energy levels in lupins, the relationship of these parameters with the nutritional value

of the meal in fish is largely unknown. Accordingly, there is a need to evaluate the level of inherent variability in the nutritional value of lupin meals, and to ascertain the relationship between protein digestibility and some easily measurable feature(s) of the grain.

An understanding of these key nutritional attributes will also improve the capacity to design constructive research and development extension in key market areas. Presently, the key competitor to the use of lupin meals in aquaculture feeds is soybean meal, both domestically and internationally. There are a range of factors influencing the perceived superiority of soy meal, such as price, supply volumes and also the consistency of the nutritional value of the meal. Therefore it is important that comparative assessments are also made against this product.

1.5 Evaluating grain protein products in aquaculture diets

As with the application of all feed resources, at some stage an assessment needs to be made of their value to their intended animal. Aquaculture feeds differ substantially from feeds for other animal sectors in their specifications, their manufacture and their delivery. Because of these differences, the application of data from other sectors is often of little relevance, as are many of the research approaches. However to resolve the questions of raw material application to aquaculture feeds, many research approaches have been attempted (reviewed by; Glencross et al., 2007). In raw material/ingredient evaluation for aquaculture diets, the three key research criteria are:

1. Defining the amount of digestible nutrients that can be derived.
2. Examining the influence of ingredient inclusion on feed intake/palatability.
3. Examining the influence of ingredient inclusion on metabolic function to define the influence of anti-nutritional factors.

Only when these key factors have been defined can the potential prospective value of an ingredient to an animal be determined (Glencross et al., 2007). Additional factors such as ingredient functionality, influences on sensory qualities of the product and the pathology associated with using certain raw materials, are additional aspects that can be considered.

Ingredient characterisation is the first part of any evaluation process. Important features such as the chemical composition, variability in composition, source and species of origin are all important factors that need to be documented so as to allow any meaningful assessment and reporting of that assessment. Detailed compositional information on test samples of all ingredients being evaluated is critical. High levels of variability between common ingredients is well recognised and this variability can affect the nutritional value of the ingredient and determination of the best strategies to assess the nutritional value of the ingredient (Jiang, 2001).

Ingredient digestibility is the measurement of the proportion of energy and nutrients that an animal can obtain from a particular ingredient through its digestive and absorptive processes. While several methods have been used to determine diet and ingredient digestibilities in aquaculture species the issue remains a contentious one. However, because most modern fish feeds are now formulated on a digestible basis it is important that this information is collected and considered (Glencross et al., 2007).

Assessment of the effects of an ingredient on diet palatability is a second key component of knowledge required about an ingredient before it can be successfully used. Palatability being defined as the combination of both attractiveness and ingestion of a diet and therefore of most relevance

to feed development. Irrespective of how digestible and available the nutrients and energy from an ingredient might be, if the ingredient reduces feed intake then it will have reduced value.

The determination of nutrient utilisation or interference with nutrient utilisation due to incorporation of any one ingredient is perhaps the most complex step in the ingredient evaluation process. This complexity is largely related to the wide variety of factors that may impact on nutrient or energy utilisation (Glencross et al., 2007).

Ingredient functionality is another crucial aspect of ingredient evaluation. Irrespective of the compositional or nutritional attributes of an ingredient, if it cannot be functionally introduced into a feed in a manner that allows it's processing in a suitable manner then it is of diminished value as a feed ingredient. Alternatively some ingredients may add additional value to a diet based on some functionality features that they contribute to a formulation. This is particularly the case with modern extruded feeds.

1.6 Project Strategy

The project has the overarching objectives of developing new, higher value markets for lupins and to also facilitate the adoption of fishmeal alternatives into aquaculture feeds in Australia. Previous projects examining these issues, while technically successful, did not deliver industry outcomes to the extent expected by each industry sector. Because of this lag in industry uptake it was decided to implement a targeted project/program to address issues across both sectors with close engagement of both industry sectors. This program assembled a large project team with a broad range of skills to address issues from grain processing, grain product development, feeds processing, nutritional evaluation, grain chemistry and grain logistics. The team engaged participants from 11 different research organisations and three industrial partners.

With the engagement of the three industrial partners a variety of grains were assessed for their potential to produce value-added products. At the request of industry a specific focus was directed towards the assessment of lupin kernel meals as a value-added grain product. However, a range of additional processes for grain value adding were examined and the key limiting factors to the production of each examined. Each of the value-added products developed was assessed for key nutritional value parameters when fed to a fish, which included digestibility and palatability assessment, and where warranted extended to growth studies with some products. The more promising products were identified for further evaluation in specific aquaculture species of shrimp and Atlantic salmon. In addition to this the influences of these value-added grain products on the processing and physical properties of the feeds into which they have been included was also evaluated.

It was also considered important to evaluate any potential nutritional limitations of the grain products in aquaculture feeds. From the results of digestibility, palatability and growth studies undertaken in assessing the new products, possible limitations to performance were further examined to define the cause of any limitations observed. Notably, potential issues with alkaloids, product variability and high soluble fibre levels in the gut of fish at high water temperatures were considered.

From this work is was proposed to provide grain producers, grain processors, aquaculture feed manufacturers and the prawn and salmon aquaculture feed industries with information about the nutritional characteristics and quality assurance criteria of grain products so that they can be marketed and used with greater confidence in aquaculture feed formulations.

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2.0 Contracted Objectives

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2.1 Introduction

This project represents a major joint initiative by the Grains Research and Development Corporation (GRDC) and the Fisheries Research and Development Corporation (FRDC). Because of this bilateral approach there are both shared and discrete interests of each stakeholder according to their industry sector requirements. In recognition of this, the key contracted objectives of the overall program are presented in terms of addressing the requirements of each industry sector. The overall the project has two fundamental objectives:

- to develop new, higher value markets for lupins
- to facilitate the adoption of fishmeal alternatives into aquaculture feeds in Australia

2.2 GRDC Objectives

However, there were three key objectives to the GRDC project component of the program. These objectives were:

- *Development of value-added lupin protein product for use in the animal feeds sector.*

  From a variety of grains, value-added products shall be developed and their manufacturing processes detailed. A range of processes will be examined and key limiting factors to the production of each identified.

- *Evaluation of the nutritional value of a range of value-added lupin protein products when fed to fish.*

  Each of the value-added products developed should be assessed for key nutritional value parameters when fed to a fish. This shall include digestibility and palatability assessment, and where warranted extend to growth studies with some products. Promising products will be identified for further evaluation in target aquaculture species.

  In addition to this the influence of these value-added grain products on the processing and physical properties of the feeds into which they have been included will also be evaluated.

- *Commercial transfer of intellectual property for development of new-product(s).*

  Outcomes of the research need to be extended to the commercial sector. This includes the facilitation of development of value-added products and subsequent assessment as needs arise. Promotional extension trips will be undertaken to key value-added grain markets.

2.3 FRDC Objectives

There were also three key objectives to the FRDC project component of the program. These objectives were:

- *To determine the nutritional value of selected grain products developed as part of the linked*

GRDC project, when included in feeds for Black tiger prawns and Atlantic salmon.
From certain new products previously identified as having potential, the digestibility, palatability and influence on growth will be assessed in Black tiger prawns and Atlantic salmon. This will allow extension of the findings from the GRDC project of the program to selected target aquaculture sectors and also allow for some cross-referencing across aquaculture species.

- *To evaluate any potential nutritional limitations of the grain products in aquaculture feeds.*
From the results of digestibility, palatability and growth studies undertaken assessing the new products, any limitations to performance will be further examined in studies targeted to defining the cause of any limitations observed. Notably, potential issues with alkaloids and high soluble fibre levels in the gut of fish at high water temperatures were perceived as possible issues.

- *To provide grain producers, grain processors, aquaculture feed manufacturers and the prawn and salmon aquaculture industries with information about the nutritional characteristics and quality assurance criteria of grain products so that they can be marketed and used with confidence in aquaculture feed formulations.*
Close collaboration between the research and the commercial sector will be facilitated to allow rapid uptake of findings and engender confidence in the research outcomes. Regular workshops and meetings will be held as part of this process to exchange information and where required, to extend it to broader audiences.

3.0 Project Outcomes

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3.1 Introduction

The research presented in this report was carried out to improve our understanding of the nutritional characteristics of a range of grain resources, but with a specific focus on lupins and their potential for aquaculture feeds. Central to this work was the objective to improve our ability to use these resources in aquaculture diets in both nutritional and functional aspects. Numerous outcomes were achieved from this research that will strengthen the position of grain products in general and lupins in particular, as ingredients to be considered and used with increased confidence by the aquaculture feed industry. The outcomes will also serve prospective lupin processor's interests in defining some of the quality criteria that will be important to the aquaculture sector. The outcomes can be generally categorised as being pertinent to either the grain sector or aquaculture feed sector. However, in some instances the distinction of which sector the outcome is targeted to is not defined, by the fact that it clearly serves the interests of both sectors.

3.2 Grain sector outcomes

- The dehulling of lupins significantly improves their overall protein content and their nutritional value to fish. With increasing dehulling efficiency a linear response in protein content is achieved with a reciprocal loss in carbohyrate content of the meal. No effect on the lipid content of the meal is observed. The extent of the protein increase varies with grain species and cultivar and is influenced by both seed protein content, the proportion of the seed as hull and the efficiency of hull removal.
- Substantial variability in the kernel meal composition of *L. angustifolius* exists. Across a collection of 75 different samples a (mean ± S.D.), protein level of 45.4 ± 3.45% on a dry basis was determined. Across all the kernel meals minimal and maximal protein levels of 36.5% and 56.7% were observed respectively. A series of the kernel meals were also produced from seed collected from three successive years production of commercial culitvars grown that the same site. From these samples substantial variability in composition was observed, with the environmental (year) effect on composition more pronounced than that of cultivar.
- The use of near-infrared spectroscopy (NIRS) was shown to be able to provide rapid and useful assessments of a range of crude composition parameters of whole grain and kernel meals. This should allow grain processors and users to rapidly and more accurately assess the actual value of discrete batches of grain products. This could provide a simple and rapid avenue for grain quality segregation and value-adding.
- Clear lupin quality criteria have been established for use of this grain in the aquaculture feed sector. Grain from which kernel meals can be produced with protein levels in excess of 42% (dry basis) constitute an effective lower protein limit for use in aquaculture feeds. The protein should be in excess of 90% digestible and alkaloid levels in the meal less than 500 mg/kg. Lignin should also be as low as possible and there is significant capacity to measure these quality parameters rapidly using NIRS and assist the grain-breeding process.

- Using both protien concentration and isolation techniques, a series of protein enriched products were prepared from *L. angustifolius*, *L. luteus* and *L. mutabilis* kernel meals. Using protein isolation methods it was possible to produce products with protein levels in excess of 80%. Protein concentration methods produced products of a lower protein content, but had a greater yield. Both yield and and protein content will be important factors in determining the commercial viability of the final products.
- Several different drying methods were examined in the production of protein concentrates and isolates. While freeze-drying proved to be a useful experimental/laboratory scale method that produced a light, low-density, friable powder, it was not considered a viable industrial scale method. For up-scaling, spray-drying and ring-drying technologies were examined with both *L. angustifolius* and *L. luteus* protein isolates. Spray-drying proved to produce good consistent product, while ring-drying proved to gum the products and not produce a useful product.
- A highly characterised sample set of lupin seed and kernel meals was collected, prepared, analysed and evaluated for their digestible energy and nutrient values. This data was then supplied to each of the collaborating commercial grain industry partners, along with samples of the seed and kernel meal, to allow the development of calibrations for chemical and nutritional properties using near infrared spectroscopy (NIRS).
- Significant adoption of the use of lupin kernel meals in aquaculture diets was instigated by Skretting Australia, the largest aquaculture feed manufacturer in Australia. This has precipitated flow-on effects leading to further adoption of lupin kernel meal use in aquaculture diets being achieved by other feed companies both domestically and internationally. Not withstanding supply and cost limitations induced by drought, increases in the use of lupin kernel meals were noted each year from 2003 to 2006.
- Drawing from the work in this project, CBH-Group and Weston Technologies have formed a joint-venture company to develop a 200,000 tonne per annum lupin kernel meal production facility. The joint-venture company, Australian Lupin Processing Pty Ltd commenced production in early 2007. The targetting of lupin kernel meals to the aquaculture market was highlighted as one of its key initiatives.
- Several smaller grain processors (e.g. Coorow Seed Cleaners) have also begun commercially producing and marketing lupin kernel meals to the aquaculture sector.

3.3 Aquaculture feed sector outcomes

- The dehulling of lupins significantly improves their nutritional value to fish. A linear increase in digestible energy value was observed, while a curvilinear response in digestible protein value was observed. This finding shows that there is significant nutritional benefit to the fish in optimising the dehulling efficiency of lupins. In terms of protein value a minor contamination with hulls is unlikely to significantly reduce the value of the protein. However, the more efficient the dehulling process the higher the overall protein content of the meal and therefore the greater its overall value.
- The influence of the lupin alkaloid gramine was shown to exert its anti-nutritonal effect through being a feed intake inhibitor. Critical threshold for tolerance to gramine intake by rainbow trout was shown to be between 100 and 500 mg/kg of diet. This provides evidence that the alkaloid levels present in Australian domestic lupin varieties are unlikely to result in anti-nutritional problems for fish. These data indicate that there is significant scope for plant breeders to increase the gramine levels in the Yellow lupin from its current very low level

to levels that will provide much better protection against aphids, without compromising the nutritional value of the kernel meal.

- Demonstration that fish can use lupin protein and energy as efficiently as fishmeal protein and energy, when diets are formulated and assessed on a digestible nutrient basis. This finding dispells the “myths” that carnivorous fish can only be effectively grown on animal derived protein sources.
- Variability in the digestible protein and energy value of the lupin kernel meals was shown to be related to kernel meal composition. Higher protein levels in the meal correlated with better protein and energy digestibility. The high protein levels also correlated with lower non-starch polysaccharide (NSP) levels in the kernel meals and this resulted in a concommitant relationship between protein, NSP and digestibility parameters. Assessment of the fibre composition of the kernel meals also showed that lignin was a key fibre class that affected protein digestibility, with higher lignin levels strongly correlating with poorer protein digestibility.
- The use of near-infrared spectroscopy (NIRS) was shown to be able to provide rapid and useful assessments of not only crude composition of whole grain and kernel meals, but also their digesitble protein and energy value. This should allow grain processors and users to rapidly and more accurately assess the actual value of discrete batches of grain products.
- The impact of variability in the digestible protein content of lupin kernel meals was assessed in two separate growth experiments. The first experiment used low-protein diets (350 g/kg) and high-inclusion levels (40%) of a low digestibility and high digestibility lupin kernel meals and soybean meal. These diets were then fed at a range of ration levels from starvation to satiety to examine both palatability and utilisation aspects of the feeds. The results demonstrated that a significant effect of the lower digestibility lupin kernel meal could be measured as an effect on growth using this design. A second experiment examined the effect of the same raw materials at lower inclusion levels (25%), in diets formulated to more typical commercial specifications (400 g/kg protein, 250 g/kg lipid). In this second experiment the effect of variability in digestible value was masked, demonstrating that under commercial equivalent conditions that variability in digestibility of lupin kernel meals would be unlikely to be observed, but that this built in margin-for-error adds significant cost to the diets.
- Preliminary assessment of both wet and dry concentrate technologies showed that there was greater potential for a wet technique to produce a viable product. Using simple formulation modelling methods it was identified that an “ideal” grain protein concentrate would have a protein content in the range of 50% to 60%. Ironically, the kernel meal from *L. luteus* already fulfills this criteria.
- Prototype protein concentrates made from *L. angustifolius* and *L. luteus* kernel meals were highly palatable and digestible when fed to either Rainbow trout and Atlantic salmon. A high degree of similarity in nutritional response of either species was noted, providing support for the use of either species as a model for the other. A comparison of faecal settlement and stripping collection methods showed that high levels of carbohydrate in the diet resulted in greater disparity between the results observed. Faecal stripping methods consistently provided more conservative estimates of the digestibility parameters.
- The influence of heat was shown to not have a negative impacts on the digestible value of lupin protein concentrates when fed to a fish. However, these heat-damaged protein concentrates were less palatable and did not sustain growth to an equivalent basis compared

to spray or freeze-dried protein concentrates. Processors need to be aware of the sensitivity of fish to heat damage in protein resources. However, the distinct nature of this heat damage, whether it is cumulative heat or critical temperature that is important, is not known.

- An improved chemical assay to measure reactive lysine assay was developed to assess nutritional damage caused by the high-temperature drying of the LPC's. This assay effectively measured the proportion of lysine within a sample that had its tertiary amino group rendered unavailable chemically. It was shown that the high-temperature drying of the LPC's resulted in an increased level of unreactive lysine, which was most likely due to chemical condensation of a carbohydrate molecule to this tertiary amino group. This means that the lysine becomes unavailable for use in protein synthesis, supporting the observations from the fish growth study.
- Comparison of the digestibility of extruded feeds and by inference, the ingredients, fed to either trout or Atlantic salmon showed that there was a high-degree of commonality in their responses to the different grain products. The strongest correlation was observed between the trout and the Atlantic salmon digestibility at 6°C. Poorest correlation was that observed between the two Atlantic salmon studies at 6°C and 15°C, though correlation between the trout and Atlantic salmon at 15°C was also not strong. The findings support that use of one species as an indicator of responses for another has some potential. However, although two of the data-sets were highly supoportive of each other, that the third was substantially different suggests that the data collection process has an important effect on the results achieved and to obtain the most viable cross-species data it is preferrable to have all experiments conducted by the same laboratory and personnel.
- Five different varieties of *L. angustifolius* kernel meal were examined for their variability in digestibility parameters when fed to Atlantic salmon. Significant variability was observed in crude protein digestibilities from each of the kernel meals. Ingredient protein digestibility ranged from 66.1% to 94.8%.
- The influence of lupin kernel meals, soybean meal and a lupin protein concentrate on gut transit in Atlantic salmon was examined using a marker replacement method. The results of this work showed that the inclusion of lupin kernel meals increased the rate of gut transit of the feed compared to the effects induced by the inclusion of soybean meal or a lupin protein concentrate.
- The inclusion of lupin kernel and soybean meal in diets for sea-water reared Atlantic salmon was examined at two inclusion levels (15% and 25%) and at two water temperatures (14°C and 18°C) to examine if there was any influence of diet raw material on temperature response. An improved feed intake and growth response was observed from fish fed the lupin kernel meal diets compared to both the fish meal based reference and the soybean meal diets. This improved performance of the lupin kernel meal diets was observed at both water temperatures. No interaction effect of temperature and ingredient was observed in the study. These findings show that lupin kernel meals have a significant advantage over soybean meal when included in diets for sea-water reared Atlantic salmon.
- The effect of yellow and narrow-leafed lupin kernel meals and protein concentrates on the gastrointestinal integrity, capacity for digestive hydrolysis, and digestibility of nutrients in Atlantic salmon were examined in fish kept at 6°C. Protein digestibility from a series of test ingredients was observed to be higher in fish at 6°C than the same diets and ingredients fed to Atlantic salmon at 15°C. Protein digestibility was highest for the *L. luteus* protein concentrate (107.7%) and lowest for the *L. angustifolius* cv Myallie kernel meal (70.5%).

- As series of gut-health related issues were observed with the different grain protein raw materials. Ulcer-like lesions were observed in the stomach of fish from all feeding groups, and this was worsened by the presence of lupin in the diet. No consistent altered morphology was observed in distal intestine of fish fed either fishmeal and lupin diets, while the distal intestine of fish fed soybean meal showed consistent and typical soybean meal-induced pathomorphological changes. The inclusion of soybean meal in the diet resulted in watery faeces and lowered the apparent digestibility of lipid, but this was not observed when feeding the lupin diets.
- The digestibility of dry matter, crude protein and energy of the yellow lupin *Lupinus luteus*, as well as of six of the new cultivars of *Lupinus angustifolius* were determined when included in diets for the black tiger prawn, *Penaeus monodon*. The apparent digestibility of the amino acids of five of the new cultivars of *L. angustifolius,* and of *L. luteus*, were also determined, a first for raw material evaluation for prawns. The apparent energy digestibility varied between 69.6% and 77.2% whereas the apparent crude protein digestibility varied between 92.7% and 96.8%. The apparent digestibility of the amino acids was similar to the apparent crude protein digestibility value. Although there was significant variability, the general consistency of the *L. angustifolius* apparent digestibility results suggests that nutritionists and feed formulators can confidently use mean apparent digestibility values for dry matter, protein and energy for kernel meals comprising of random mixtures of cultivars.
- The performance of black tiger shrimp, *Penaeus monodon* when fed one of seven of the new cultivars of *Lupinus angustifolius* or solvent-extracted soybean meal was examined in a series of growth studies. In each experiment the growth rate of shrimp fed the diets containing lupin kernel meal or soybean meal was as good as, or better than that obtained with the fish meal based basal diet. Survival in all experiments was high (mean ~90%). These findings have demonstrated that lupin kernel meal can be used to replace at least 40 % of the fishmeal protein in diets for *P. monodon*, and that the new cultivars perform equally to solvent-extracted soybean meal when used on a protein-equivalent basis. From the amino acid analysis of the diets used in the experiments, it appears that that the reported requirements of juvenile *P. monodon* for methionine significantly overestimate the true requirements.
- Because prawns have a different sensory system to that of fish, the effect of the lupin alkaloid, gramine, when included in a feed for the black tiger prawn, *Penaeus monodon* was examined. The daily feed intake, growth rate and survival of the prawns was not affected by the concentration of gramine in the feed over the range of concentrations examined (0 to 902 mg/kg of feed, as used). High levels of gramine did significantly reduce feed intake in the first 15 min after distribution of the feed. But, thereafter over the following 6 h that were closely monitored, feed intake did not appear to be affected by gramine inclusion level. It was noted that gramine leached from the feeds quite rapidly with about 20% of the gramine lost in the first hour. This leaching observation may explain the observed responses of the prawns to this alkaloid.
- From common lupin kernel meals studied in Rainbow trout, prawns and Atlantic salmon a comparison of the digestibility of protein and energy was made among the three species. No significant relationships were observed among any of the species. It is suggested that limited variability observed in digestibility values of the tested lupin kernel meals made it difficult to define possible inter-relationships in these parameters. Differences in experimental methods and laboratory routines also make direct comparison difficult.
- Lupin kernel meal inclusion in an extruded pellet resulted in an increase in pellet hardness, bulk density and sink rate with increasing lupin inclusion. The relationship was generally

curvilinear, with maximal responses occuring at around 20% inclusion. Extruded pellet expansion and vacuum oil uptake were generally reduced with increasing lupin inclusion. Water retention in the extrusion mash was also enhanced by the inclusion of increasing levels of *L. angustifolius*, *L. luteus* or soybean meal. This higher water retention in the mash has benefits in reducing wear on the extruder and also increasing the rate at which gelatinisation of the starch in the diet occurs.

- Significant varibility in diet extrusion features was observed as a function of different lupin varieties/culitvars and also the actual species of feed grain being included in a diet. The inclusion of lupin kernel meals (from either *L. angustifolius* or *L. luteus*) was shown to increase bulk density, sink rate and pellet hardness and decrease vacuum oil uptake and pellet expansion, at a different degree than that achieved by a similar inclusion of soybean meal. However, the degree to which each factor was affected varied depending on grain product and its inclusion level.
- A series of studies were undertaken to examine the composition, digestibility and palatability to rainbow trout of different types of value-added grain products. Details of each product and their assessment were conducted on a commercial-in-confidence basis and as such no details will be provided. A total of eight products from both CBH-Group and Weston Technologies were evaluated over a two-year period.
- Skretting Australia, the largest aquaculture feed manufacturer in Australia have broadly adopted the use of lupin kernel meals across their product range. The adoption of the raw material has also spread further within this multinational group, with companies within the Skretting group in Norway, Japan and Chile also adopting the use of lupin kernel meals. Other feed companies in Australia, and internationally, are now following the lead of Skretting and also commencing adoption of the use of lupin kernel meals.

4.0 The influence of dehulling efficiency on the digestible value of lupin (*Lupinus angustifolius*) kernel meal when fed to rainbow trout (*Oncorhynchus mykiss*)[a]

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Abstract

A single crop batch of *Lupinus angustifolius* (cv. WALAN2173M) seed was processed to produce both a seed meal and also dehulled to produce a pure kernel meal. A series of blends were prepared from the seed and kernel meals (100%:0%, 83%:17%, 67%:33%, 50%:50%, 33%:67%, 17%:83%, 0%:100%, respectively). The various blends were then used to determine the relative nutritional effects of varying degrees of dehulling efficiency. The digestible value of these neat and blended meals were compared when fed to Rainbow trout (*Oncorhynchus mykiss*) using the diet-substitution method (70% reference: 30% test ingredient). Stripping methods were used to collect faecal samples for the determination of digestible energy and nutrient values of the neat and blended meals being tested. Significant improvements were observed for each of dry matter, energy and protein digestibilities with increasing dehulling efficiency. The relationship between dry matter digestibility and kernel meal proportion was curvilinear and described by the equation: $y = -0.00001x^2 + 0.00299x + 0.39752$. Dry matter digestibility for the 100% kernel meal was 59.8%. The relationship between protein digestibility and kernel meal proportion was curvilinear and described by the equation: $y = -0.00002x^2 + 0.00395x + 0.81914$. Protein digestibility for the 100% kernel meal was 101.7%. The relationship between energy digestibility and kernel meal proportion was linear and described by the equation: $y = 0.0016x + 0.4877$. Energy digestibility for the 100% kernel meal was 65.1%. The findings of this study demonstrate that there are significant benefits from using kernel meals over seed meals, beyond the general increased crude levels of protein and energy gained.

4.1 Introduction

Modern nutrient-dense diets for aquatic species have limited formulation flexibility to accommodate large amounts of non-useful nutritional content (e.g. fibre or ash). Because of this, many feed grain resources are not viable alternatives, despite having reasonable protein or energy digestibilities. To address this limitation one option is to process some grain varieties to produce protein enhanced products. Such protein concentrated products also allow some flexibility to remove potential anti-nutritional factors found in feed grains (Glencross et al.,

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2003a). Dehulling is a mechanical procedure used to process some legumes and oilseeds. The process abrades the grain to remove the seed coat (hull) from the seed kernel. Following this aspiration is used, which using density differentiation, allows for some separation of the hull from the seed kernels. Under laboratory conditions it is reasonable to obtain a pure sample of dehulled kernel material for evaluation (Booth et al., 2001; Allan and Booth, 2004; Glencross and Hawkins, 2004). However, under commercial conditions 100% efficiency in the extraction of hulls from the dehulling process is unviable.

There is a considerable volume of work on the nutritional value to salmonids of grain products produced from soybean, peas and lupins, where the grain has been processed to produce a dehulled product (Kaushik et al., 1995; Refstie et al., 1998; Carter and Hauler, 1999; Burel et al., 2000; Glencross and Hawkins, 2004; Glencross et al., 2004a; 2004b). Additional work with Silver perch (*Bidyanus bidyanus*) has further compared the specific effects of whole-seed and dehulled preparations of a range of legumes, including *Lupinus angustifolius* lupins (Booth et al., 2001; Allan and Booth, 2004). Both of these works have showed that there are clear advantages to dehulling lupins, with significant improvements in dry matter and energy digestibilities and minor improvements in protein digestibility, albeit not significant ones. However the effect of variable efficiency of the lupin dehulling process on the sample composition and the concomitant response of digestibility of those meals by a fish species has not been explored. This aspect has important implications with regards to the application of this feed grain when processed using industrial scale operations where 100% dehulling efficiency is unlikely to be obtained.

This study examines a range of hull concentrations remaining in the meals, representing variable dehulling efficiencies. These different meals being reflective of the variable dehulling efficiencies potentially resulting from industrial scale dehulling of this feed grain. From this the effects on meal composition and their digestible value when fed to Rainbow trout, *Oncorhynchus mykiss* are determined.

4.2 Materials and Methods

4.2.1 Ingredient and diet development

A single crop batch of seed of *Lupinus angustifolius* (cv. WALAN2173M) was used in this study. Samples of the seed were either milled or dehulled and milled to create stock samples of seed meal or kernel/dehulled meal. The pure dehulled sample was prepared using abrasive dehulling, followed by differential density aspiration to separate hulls and kernels, before a final manual removal of any remaining hull material. A series of seven blends between the two different stock samples were created by adding different amounts of each meal to each other with vigorous mixing to create a series of blends between 100% seed meal and 100% kernel meal. The composition and source of all of the ingredients used are presented in Table 4.1. Each of the test ingredients was thoroughly ground such that they passed through a 750 μm hammer mill screen.

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 4.2). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 4.2). Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst

mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. The diet formulations and source of all of the ingredients used is presented in Table 4.2. Composition of all experimental diets is also presented in Table 4.2.

4.2.2 Fish handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 L). Freshwater (salinity < 1 PSU) of 16.0 ± 0.1°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 15 trout of 257 ± 34.4 g (mean ± S.D.; n = 40). Treatments were randomly assigned amongst 24 tanks, with each treatment having three replicates.

Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600 each day. The trout were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was taken to ensure that the faeces were not contaminated by urine or mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and stored in a freezer at -20°C. Stripped faeces were collected during 0800 to 1000 over a four-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples from different days were pooled within tank, and kept frozen at -20°Cbefore being freeze-dried in preparation for analysis.

4.2.3 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia and Animal Health Laboratories, South Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by McQuaker et al., (1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Amino acid composition of samples was determined by an acid hydrolysis prior to separation via HPLC. The acid hydrolysis destroyed tryptophan making it unable to be determined. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the method of Folch et al. (1953). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 4.4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%.

4.2.4 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Effects of ingredient on digestibility of dry matter, protein and gross energy in each of the ingredient were examined by one-way ANOVA (Table 4.3). Curve fitting of both linear and polynomial regressed relationships was undertaken using both Microsoft Excel and Statitistica v6. Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

4.3 Results

4.3.1 Ingredient composition

The lupin-based ingredients produced in this study had a range of compositions (Table 4.1). The dehulling process had a clear significant effect of increasing protein content and reducing carbohydrate content of the meal. No significant influence of dehulling on fat content of the meals was observed. Changes in absolute amino acid composition were consistent with the protein concentration effect of the dehulling process, but no specific changes in relative amino acid concentrations were observed.

4.3.2 Diet digestibility

Apparent dry matter digestibilities of the diets significantly increased with increasing dehulling

efficiency, although the effects varied numerically only from 69.2% to 82.8% for the 100% seed meal and reference diets respectively (Table 4.3). Apparent protein digestibilities of the diets also increased with increasing dehulling efficiency, although the numerical effect was minimal, varying only from 90.5% to 92.0% for the 100% seed meal and 100% kernel meal diets respectively (Table 4.3). Despite this limited variation the consistency of the data was still robust enough to identify significant effects between these levels of protein digestibility differences. Apparent energy digestibilities of the diets also significantly increased with increasing dehulling efficiency, although the effects varied numerically only from 78.5% to 90.2% for the 100% seed meal and reference diets respectively (Table 4.3).

4.3.3 Ingredient digestibility

Apparent dry matter digestibilities of the meals significantly improved with increasing dehulling efficiency (Table 4.3 and Figure 4.1). Regression analysis of the relationship between dehulling efficiency and apparent dry matter digestibility supported that this was a linear relationship (R^2 = 0.8772) (Figure 1). With a pure (100%) kernel meal, an apparent dry matter digestibility of 59.8% was determined for the ingredient at a 300 g/kg inclusion level. This contrasted the pure (100%) seed meal, which had an apparent dry matter digestibility of 39.1%, which was also determined for the ingredient at a 300 g/kg inclusion level.

Apparent protein digestibilities of the meals were significantly improved with increasing dehulling efficiency (Table 4.3 and Figure 4.2). Regression analysis of the relationship between dehulling efficiency and apparent protein digestibility supported that this was a clear second-order polynomial relationship (R^2 = 0.9437) with a reduction in apparent protein digestibility with decreasing efficiency in the dehulling process (Figure 4.2). However, ANOVA and a post-hoc LSD analysis supported that protein digestibility is only significantly reduced below a 50% dehulling efficiency. With a pure (100%) kernel meal, an apparent protein digestibility of 101.7% was determined for the ingredient at a 300 g/kg inclusion level. This contrasted the pure (100%) seed meal, which had an apparent dry matter digestibility of 83.3%, which was also determined for the ingredient at a 300 g/kg inclusion level.

Apparent energy digestibilities of the meals significantly improved with increasing dehulling efficiency (Table 4.3 and Figure 4.3). Regression analysis of the relationship between dehulling efficiency and apparent energy digestibility supported that this was a linear relationship (R^2 = 0.9652) with no improvement in regression with the use of a second-order polynomial function effect (Figure 4.3). With a pure (100%) kernel meal, an apparent energy digestibility of 65.1% was determined for the ingredient at a 300 g/kg inclusion level. For the pure (100%) seed meal, an apparent energy digestibility of 49.4%, was determined for the ingredient, also at a 300 g/kg inclusion level.

The ingredient digestibility of carbohydrates was determined in two separate manners, both based on inferred measurements as no direct measurements of the highly variable carbohydrate composition were undertaken. In the first method the carbohydrates were determined based on the difference between total dry matter minus protein, fat and ash (all dry matter corrected) (Figure 4.4a). In the second method the energetic contribution of carbohydrates based on the total digestible energy value of the ingredient minus the energetic contributions of the determined digestible protein and fat, divided by the energetic value of carbohydrate was calculated (Figure 4.4b). This assumed energetic constant values for protein, fat and carbohydrate of 23.6, 38.5 and 17.3 MK/kg DM respectively.

4.4 Discussion

There have been numerous studies examining the digestible value of lupins when fed to a variety of fish species (Burel et al., 1998; Booth et al., 2001; Glencross and Hawkins, 2004). Most of these studies have focussed on the nutritional assessment of lupin kernel meals, which are now being used in significant amounts in modern commercial extruded feeds (Glencross, 2005). Early studies often examined the nutritional value of whole-seed lupin meals (De la Higuera et al., 1988; Morales et al., 1994; Gomes et al., 1995; Robaina et al., 1995). What comparisons there have been between the whole seed and kernel meal varieties have shown substantial differences in nutritional value (Booth et al. 200X). While Booth et al. (2001), compared the effects of dehulled versus whole seed lupins when fed to Silver perch (*Bidyanus bidyanus*), the omnivorous dietary nature of this species makes extrapolation of this work to other more carnivorous species less relevant. Furthermore, the influence of variability in the dehulling process had also not been assessed for any fish species. This study is the first to examine the digestibility response of a fish to increasing levels of lupin dehulling efficiency. This is important because although a 100% pure kernel meal is achievable on an experimental scale it is unlikely to be ever achieved commercially. Therefore this study assesses the consequences of different degrees of dehulling efficiency that will cover the spectrum of all potential industrial dehulling operations.

4.4.1 Ingredient composition

The changes noted of the composition of the lupin meal with increasing dehulling efficiency clearly show the benefit of processing the grain. Principally there was an increase in the meal protein content and the lower levels of non-starch polysaccharide carbohydrates with increased dehulling efficiency. Limited effect on the lipid content of the meals was noted. With the consistent lipid levels, increase in protein and decrease in carbohydrates there was, accordingly an increase in gross energy density. This effect is consistent with most other comparisons of whole seed and kernel meals (Petterson, 1999; van Barneveld, 1999; Booth et al., 2001).

The particular variety of lupin used in this study (WALAN2173M) is at the time a non-commercially released variety, but the extent of the potential increase in protein achievable with this variety is only matched by the lupin species *L. luteus* (Glencross and Hawkins, 2004; Glencross et al., 2004b). This feature alone makes this a highly valuable variety of *L. angustifolius*, especially if one were to simply assume even a linear protein to value basis. This variety will be particularly suited to aquaculture feed applications for both its compositional and digestible features.

4.4.2 Diet digestibility effects

The methods used in this study rely on the assessment of the digestibility of a reference and a series of test diets to determine the component digestibilities of the test ingredients (Aksnes et al., 1998). This method compounds potential errors and also assumes additivity of both the test and reference diet components. However recent studies have shown that raw materials with a significant complex carbohydrate content have potential interactive effects with other key nutrients in the diet (Glencross et al. 2005). Because of this although diet digestibilities are always within the realms of realistic values the potential for nutrient digestibility values greater than 100% or less and 0% are realistic possibilities. Despite these complexities the digestibilities of the diets resulted in a highly consistent pattern with respect to the inclusion of the test ingredients.

The digestibility of protein among the diets was highly consistent at around the 90% range though increased with the inclusion of more efficiently dehulled lupin kernel meals. The variability in the dry matter and energy digestibilities were more pronounced than that of the protein. This perhaps reflects the poor ability of the fish to digest the carbohydrate contents of the lupins and indeed even a potential interactive effect between the lupin carbohydrate fraction and that of the wheat. It was noted that crude carbohydrate digestibility was significantly reduced with the inclusion of any of the lupins meals. Given that lupins contain negligible levels of starch and that the hull is mostly cellulose and hemicellulose, then this effect is understandable (Petterson, 1999).

4.4.3 Ingredient digestibilities and nutritional value

Significant improvements in most digestible parameters were observed with increasing levels of dehulling efficiency of the lupins. Significant improvements were observed for each of dry matter, energy and protein digestibilities with increasing dehulling efficiency. These effects are consistent with earlier work examining different varieties of *L. angustifolius* that also had increasing protein levels (Glencross et al. 2003b). However, it maybe possible that that study also partially reflects different levels of dehulling efficiency as two of the varieties tested were the same, but differed in both compositional and digestible values.

For both the apparent dry matter and energy digestibilities of the meals there was a significant improvement on a linear basis with increasing dehulling efficiency (Table 4.3 and Figure 4.1 and 4.3). Comparison of the apparent digestibility of dry matter and energy in this study is highly consistent with those observed in other studies on the same feed grain species (Glencross and Hawkins, 2004; Glencross et al. 2005). These observations are consistent with those of Booth et al. (2001) who noted an improvement in digestibility of dry matter from 50.3% to 67.6% and an improvement in energy digestibility from 59.4% to 74.0%. Additional studies by Allan and Booth (2004) also showed similar effects with improvements in digestibility of dry matter from 44.1% to 57.6% and an improvement in energy digestibility from 53.1% to 64.2%. Based on the findings from the present study it would be reasonable to assume that the nature of these improvements is linear with Silver perch also. However, the substantial variations in digestibility values presented by the two studies poses the question as to possible differences in dehulling efficiency of the samples used or the possible effects of genotype and/or environmental influences on digestible value of this feed grain (Booth et al., 2001; Allan and Booth, 2004).

Apparent protein digestibilities of the meals improved in a clear second-order polynomial relationship, with a reduction in apparent protein digestibility with decreasing efficiency in the dehulling process (Figure 4.2). However, above a 50% dehulling efficiency there was no significant improvement in the protein digestibility of the lupin meals. This supports that from a protein digestibility basis that the presence of excess cellulose and hemicellulose from the hulls does not reduce the protein digestibility of the meals. Given that the hull has negligible protein content and contains protein that is likely to be highly bound, and provided that the physical barrier is minimised between the protein and carbohydrate content of the meal, then such a digestibility result is clearly explainable. These observations of the effect of dehulling on protein digestibility contrast those of Booth et al. (2001) and Allan and Booth (2004), both of who reported negligible improvements in protein digestibility with dehulling. The settlement faecal collection method used by these workers and/or the omnivorous nature of the fish used may explain some aspects of these differences compared to the more carnivorous fish species used in the present study (Glencross et al., 2005).

The observations, albeit indirect, of the carbohydrate digestibility of the lupin meals pose some interesting questions. It is well known that the carbohydrate complexity of the kernels of lupins is substantially greater than that of the hulls (Carre et al., 1985; Cheung 1990). What this also shows is that as the relative concentration of these carbohydrates increases then their interactive effect on the total digestibility of carbohydrates and energy in the diet is also increased. In most cases this energetic effect is largely offset by the higher contribution of protein energy value from the kernel meals and the enhanced lipid digestibility that is also observed with the inclusion of these raw materials (Glencross et al., 2005). This observation of interactive, and thereby non-additive effects is counter to some of the primary assumptions by which these digestibility effects are studied. These observations are consistent with earlier such observations and comments also made on the interactive nature of plant based raw materials (Glencross et al., 2004a; 2005). This is clearly an area that requires a more in depth evaluation to determine the specific nature of these interactive effects among carbohydrate classes.

4.4.4 Conclusions

The findings of this study confirm that there are compositional and nutritional benefits to aquaculture diets from the dehulling of lupins. When assessed using a range of digestibility parameters, each improved with an increased level of dehulling efficiency. However, with the exception of energy digestibility, most improvements were curvilinear in nature. This supports that minor inefficiencies in dehulling are unlikely to significantly diminish the digestible protein or dry matter value of these feed grains. However, the more efficient the overall dehulling process the more valuable the feed grain will be from all assessed digestible parameters and efforts to obtain the purest kernel meals will prove to be beneficial. The exception to this is the observation of the effect of the carbohydrate content of lupin kernels on their digestibility. While a larger portion of the carbohydrates present as cellulose and hemicellulose appear to not present much of a negative influence, when the proportional content of more complex non-starch polysaccharides are present then a negative interactive effect with starch is apparent. The specific nature of this interaction requires further investigation.

4.5 References

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Table 4.1 Nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[a] Wheat	[b] Seed meal (0 : 100)	Blend-1 (17 : 83)	Blend-2 (33 : 67)	Blend-3 (50 : 50)	Blend-4 (67 :33)	Blend-5 (83 : 17)	[c] Kernel meal (100 : 0)
Dry matter content (g/kg)	931	905	915	915	914	913	915	911	910
Crude protein	749	142	365	394	417	429	445	471	492
Crude fat	87	24	66	70	71	79	78	72	96
Ash	161	11	31	31	31	31	30	30	31
Phosphorus	28	2	3	3	4	4	4	4	4
Gross energy (MJ/kg DM)	20.5	18.4	20.1	20.1	20.6	20.7	20.8	21.0	21.2
Arginine	39	7	75	86	85	90	98	104	109
Cysteine	9	4	9	10	10	11	12	12	13
Histidine	18	1	5	6	6	6	6	6	7
Isoleucine	33	5	13	14	14	15	16	17	18
Leucine	60	10	0	0	0	0	0	0	0
Lysine	51	5	40	46	46	48	51	54	58
Methionine	26	2	13	11	15	17	16	18	18
Phenylalanine	30	6	13	15	15	16	16	17	17
Threonine	37	5	24	27	27	28	30	32	34
Valine	39	7	75	86	85	90	98	104	109

[a] Wheat and Fish meal: Chilean anchovy meal, Skretting Australia, Cambridge, TAS, Australia.

[b] Wholeseed *L. angustifolius* cv WALAN2173M, Department of Agriculture, South Perth, WA, Australia.

[c] Dehulled *L. angustifolius* cv WALAN2173M, Department of Agriculture, South Perth, WA, Australia.

Table 4.2 Formulations of the experiment diets (all values are g/kg).

	Reference Diet	Seed meal (0 : 100)	Blend-1 (17 : 83)	Blend-2 (33 : 67)	Blend-3 (50 : 50)	Blend-4 (67 :33)	Blend-5 (83 : 17)	Kernel meal (100 : 0)
Ingredient								
Fishmeal	700.0	490.0	490.0	490.0	490.0	490.0	490.0	490.0
Fish oil	150.0	105.0	105.0	105.0	105.0	105.0	105.0	105.0
L. angustifolius seed meal	0.0	300.0	250.0	200.0	150.0	100.0	50.0	0.0
L. angustifolius kernel meal	0.0	0.0	50.0	100.0	150.0	200.0	250.0	300.0
Wheat flour	144.0	100.8	100.8	100.8	100.8	100.8	100.8	100.8
Vitamin and mineral premix	5.0	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1.0	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Diet composition as analysed								
Dry matter	949	950	960	951	957	959	963	944
Protein	521	470	481	490	505	510	521	526
Fat	217	183	181	179	187	186	189	180
Carbohydrate*	134	250	241	233	211	206	193	195
Phosphorus	19	14	14	14	14	14	14	14
Ash	128	97	97	98	97	98	98	99
Gross Energy	23.2	22.5	22.3	22.5	22.5	22.7	22.6	22.8

[a] From *L. luteus* (yellow lupins).

[b] From *L. angustifolius* (Sweet lupins). Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

* Carbohydrate determined as dry matter minus protein, fat and ash.

Table 4.3 Digestibility (%) specifications of diets and test ingredients and digestible nutrient content (g/kg DM, unless otherwise detailed) of the test ingredients as determined using stripping faecal/digesta collection methods.

Nutrient	Reference Diet	Seed meal (0 : 100)	Blend-1 (17 : 83)	Blend-2 (33 : 67)	Blend-3 (50 : 50)	Blend-4 (67 :33)	Blend-5 (83 : 17)	Kernel meal (100 : 0)	Pooled SEM
Diet Digestibility									
Dry matter	0.828	0.692	0.703	0.722	0.725	0.721	0.752	0.744	0.005
Energy	0.902	0.785	0.791	0.804	0.810	0.804	0.821	0.824	0.004
Protein	0.917	0.905	0.906	0.916	0.917	0.913	0.920	0.920	0.001
Ingredient Digestibility									
Dry matter	-	0.391	0.454	0.497	0.525	0.514	0.634	0.598	0.009
Energy	-	0.494	0.498	0.553	0.572	0.580	0.617	0.651	0.009
Protein	-	0.833	0.860	0.917	0.999	0.982	1.026	1.017	0.008
Digestible Nutrients									
Dry matter	-	358	415	454	480	471	578	544	-
Energy (MJ/kg DM)	-	9.9	10.0	11.4	11.8	12.0	13.0	13.8	-
Protein	-	304	339	382	428	437	483	500	-

Different superscripts within rows indicate significant differences between means among ingredients, but not between nutrients or Diet/Ingredient assessment ($P < 0.05$).

Digestible nutrient values are calculated based on ingredient composition (Table 1) and ingredient apparent digestibility coefficients (Table 4). Where apparent digestibility coefficients were greater than 100%, an absolute digestibility of 100% was assumed for practicality reasons.

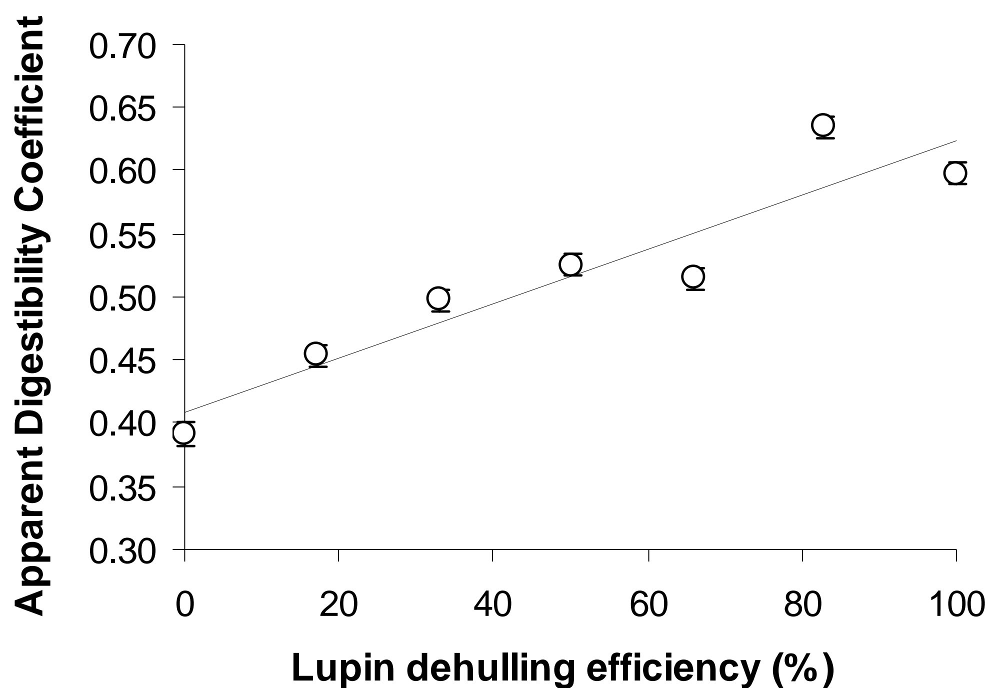


Figure 4.1 Influence of dehulling efficiency on apparent digestibility of dry matter of a lupin kernel meal when fed to rainbow trout. A significant ($P < 0.05$) increase in dry matter digestibility was observed with increasing dehulling efficiency This relationship was best described by a linear function of: $y = 0.0021x + 0.4093$, $R^2 = 0.\ 0.8772$.

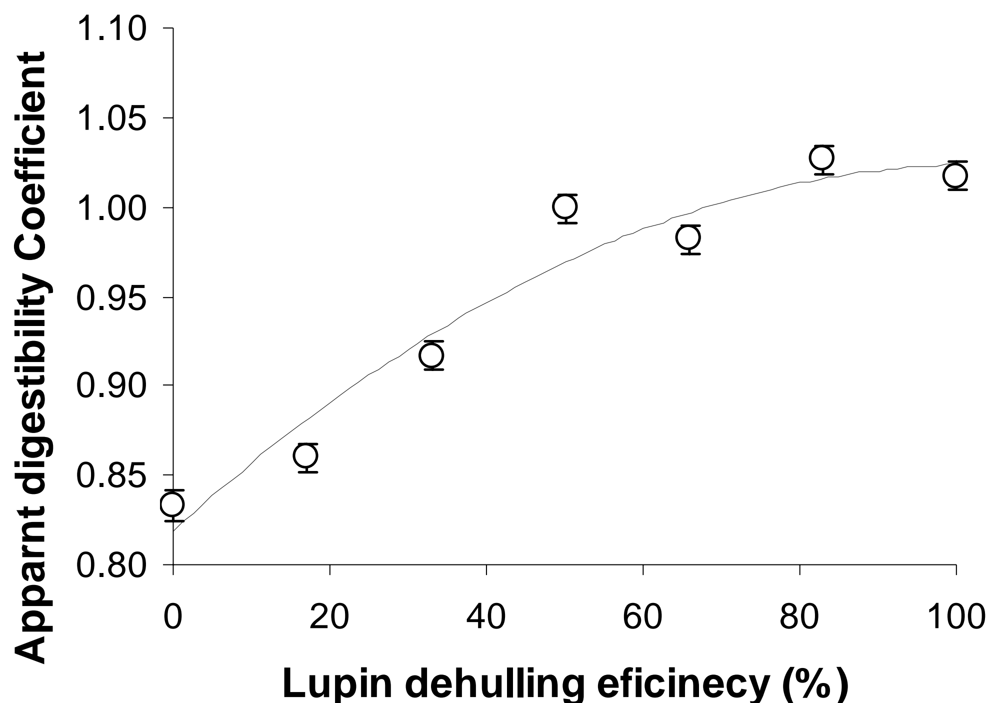


Figure 4.2 Influence of dehulling efficiency on apparent digestibility of protein of a lupin kernel meal when fed to rainbow trout. A significant ($P < 0.05$) increase in protein digestibility was observed with increasing dehulling efficiency. This relationship was best described by a polynomial function of: $y = -0.00002x^2 + 0.00395x + 0.81914$, $R^2 = 0.9437$.

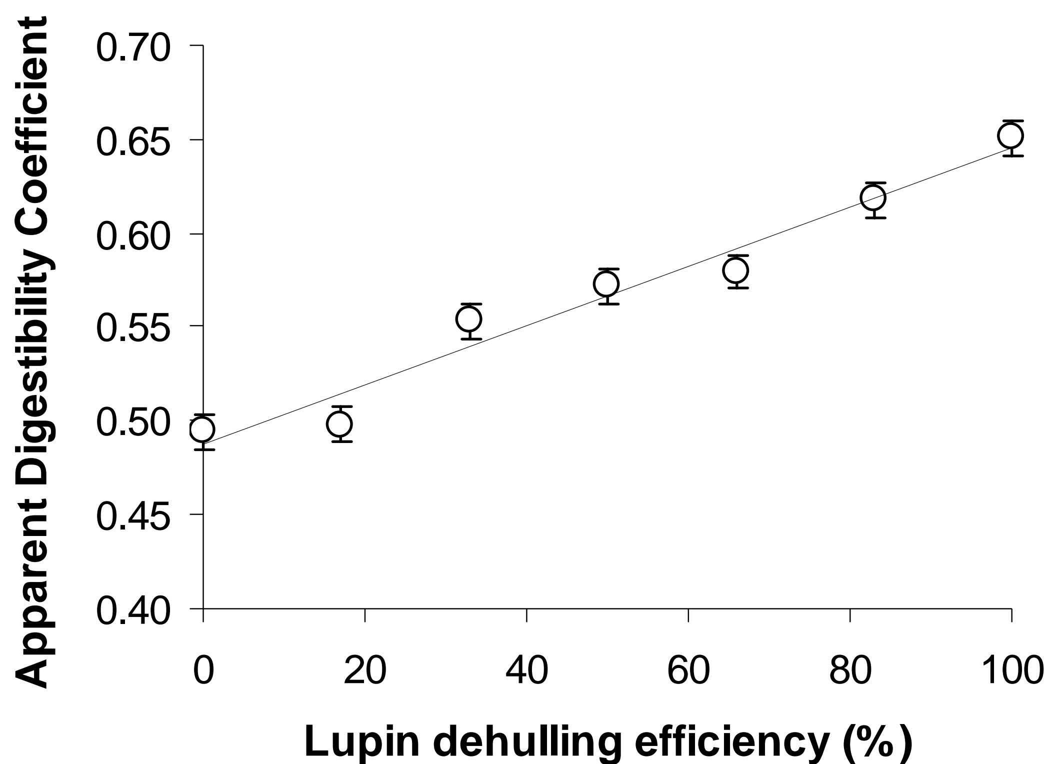
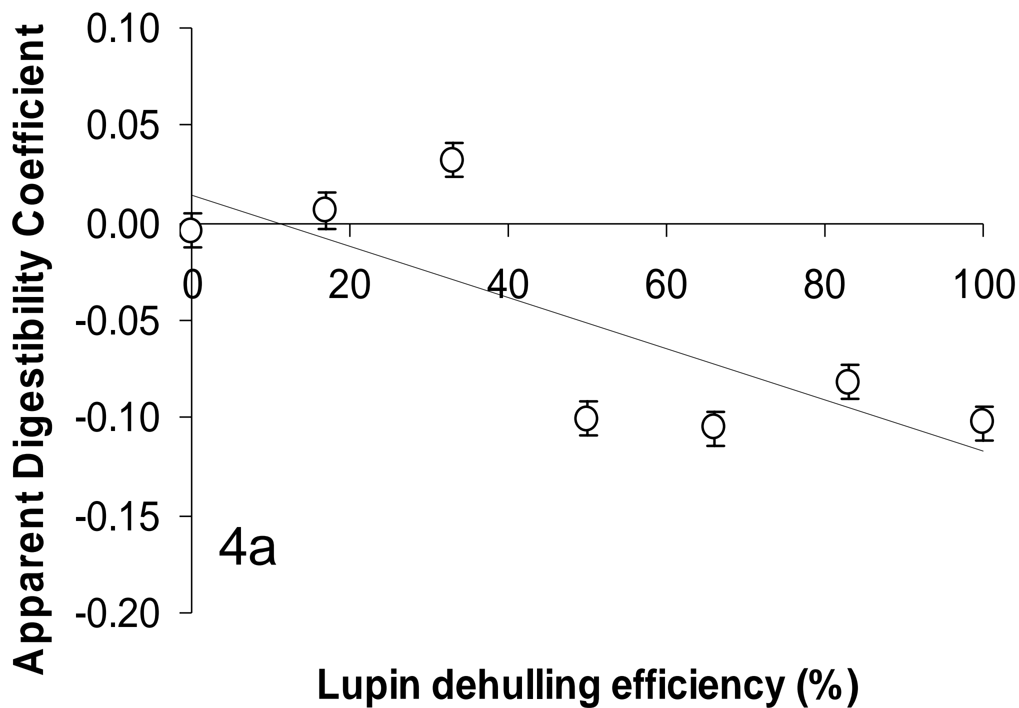


Figure 4.3 Influence of dehulling efficiency on apparent digestibility of energy of a lupin kernel meal when fed to rainbow trout. A significant ($P < 0.05$) increase in protein digestibility was observed with increasing dehulling efficiency. This relationship was best described by a linear function of: $y = 0.0016x + 0.4877$, $R^2 = 0.9652$.



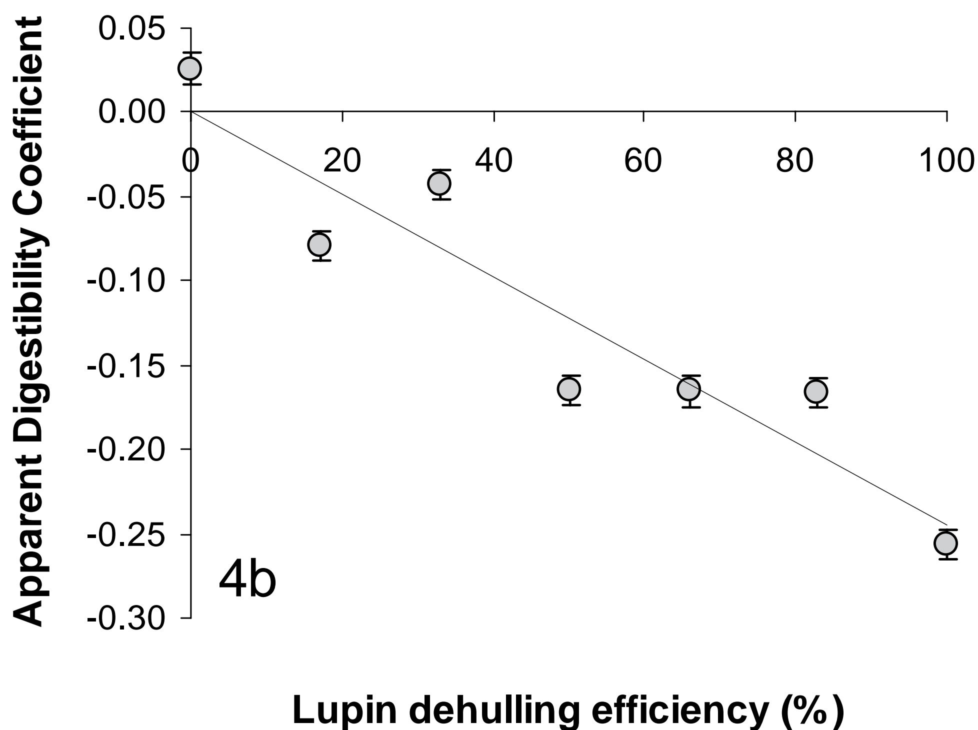


Figure 4.4 **a and b.** Based on the mass-balance contribution of carbohydrate (open circles) to the total dry matter of each test ingredient a significant ($P < 0.05$) decrease in carbohydrate digestibility was observed with increasing dehulling efficiency. This relationship was best described by a linear function of: $y = -0.0013x + 0.0144$, $R^2 = 0.6192$.

Based on the energetic contribution of carbohydrate (gray cricles) to the total energy digestibility of each test ingredient a significant ($P < 0.05$) decrease in carbohydrate digestibility was observed with increasing dehulling efficiency. This relationship was best described by a linear function of: $y = -0.0025x + 0.0013$, $R^2 = 0.8724$.

5.0 A comparison of the effect of diet extrusion or pelleting on the digestibility of grain protein products when fed to rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study examined the effect of the extrusion process on the digestibilities of whole diets and also the component test ingredients when fed to rainbow trout. Six diets were prepared using either a screw-press or an extruder based on the same batches of raw materials and formulations in each case. Correlations between diets were highly significant for all four parameters examined of dry matter, nitrogen, energy and the sum of amino acids. The correlations showed that extrusion significantly improved the energy digestibility of the diets but effects on the other parameters were negligible. Correlations between ingredients for energy and dry matter digestibilities were highly significant, but correlations between the digestibility of nitrogen and the sum of amino acids were poor. The ingredient correlations also showed that extrusion improved the digestible energy value of the test ingredients (e.g. AD_E of 70% when screw-pressed, but AD_E of 80% when extruded), but any improvement in the dry matter digestibility was nominal and no advantages were gained for protein digestibility. The results of this study show that diet digestibility responses obtained from screw-press manufactured diets provide a proportional, but not necessarily direct indication of the responses achieved from extruded diets. The ingredient digestibilities showed that while dry matter and energy digestibilities are also proportional that nitrogen and the sum of amino acid digestibilities are not proportional between the two diet manufacturing methods. Observations of pellet stability *in vivo* showed distinct differences between the reference, *L. angustifolius* cv. Myallie kernel meal and soybean meal test diets. Changes in pellet integrity were noted after 2, 4 and 8 h. Pellet integrity after 8 h was best in the reference diet and worst in the soybean meal diet. The soybean meal diet lost its structural integrity quicker than that of the reference and *L. angustifolius* cv. Myallie kernel meal diets. The specific nutritional implications of these observations need to be more fully explored.

5.1 Introduction

Modern nutrient-dense diets for most fin-fish species tend to be manufactured using a technique referred to as extrusion processing. In this process a mixture of raw materials are compressed through barrel by a screw whilst heat and steam are applied to the raw materials as they pass along the length of the barrel. At the end of the barrel the mixture, referred to as the mash, is extruded through a small aperture known as the die. In most extrusion techniques used in fish feed production a certain amount of starch is added to the mixture. This has the effect of when the mash

is extruded through the die that the release of pressure and heat causes the starch to expand and gelatinise (Shankar and Bandyopadyay, 2005). This starch expansion along with some interactions among the proteins in the mash is what gives the product its principle binding strength.

It is recognised that extrusion has dramatic effects on starch chemistry compared to less aggressive feed processing techniques such as steam-pelleting and screw-press technologies. The gelatinisation and expansion of the starch also increases its nutritional value through an increase in the digestibility of the starch to most fish species (Bergot and Breque, 1983; Jeong et al. 1991). However, it is not known whether extrusion will also affect the nutritional value of other raw materials such as lupins. Studies examining the effect of extrusion of lupins themselves, prior to inclusion in diets for fish, have suggested that significant gains are achieved (Bangoula et al., 1993). However, this has not been confirmed and reasons for why such a benefit occurs have not been identified, as virtually no starch is present in lupin seeds. Other studies with raw materials, like soybean meals, have shown benefits through extrusion of both the raw material and also when they are included un-pre-extruded in a diet that is subsequently extruded. This is generally believed to be because of the heat denaturing effect on some of the anti-nutritional factors in this raw material, like protease inhibitors and lectins (Refstie et al., 1998; Francis et al., 2001).

There is a considerable volume of work on the nutritional value to salmonids of grain products produced from soybean, peas and lupins in both extruded and un-extruded diets (Kaushik et al., 1995; Refstie et al., 1998; Carter and Hauler, 1999; Burel et al., 2000; Glencross and Hawkins, 2004; Glencross et al., 2004a; 2004b). Additional work with Silver perch (*Bidyanus bidyanus*) has further compared the specific effects of pre-extrusion of a range of legumes (Allan and Booth, 2004). Most of these works have shown that there are clear advantages to extruding some raw materials, with improvements in dry matter and energy digestibilities, but notably the ingredients that are improved tend to be ones with a high starch content and/or significant levels of heat-labile anti-nutritional factors.

This study examines a comparison in the digestible value of diets and their component test ingredients when the diets are manufactured using either extrusion or screw-press pelleting technology. The effects of these processing factors on the digestible values were examined based on the diets being fed to rainbow trout, *Oncorhynchus mykiss*.

5.2 Materials and Methods

5.2.1 Ingredient and diet development

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 5.1). A 1500 kg batch of a basal mash was prepared from a single batch of ingredients and thoroughly mixed and milled through a 750 µm hammermill, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 5.1). The composition of each test and basal mash ingredient is presented in Table 5.2. The basal diet was prepared without the addition of any test ingredient.

Diets were processed by either laboratory-scale screw-press methods using a pasta making machine (Italpast, Fidenza, Itlay) or extrusion through a laboratory scale Wenger X185 extruder

(Wenger, Sabetha, KA, United States) at the Australasian Experimental Stockfeed Extrusion Centre (AESEC). All screw-pressed diets were made using the same methods. Diets made on the screw-press were formed with the addition of water (about 30% of mash dry weight) to the dry mash (including oils) whilst mixing to form an agglomerated mash. The actual amount of water added varied according to each test ingredient but was added to an amount that caused particle agglomeration within the mixing bowl. The agglomerated mash was subsequently screw pressed through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven (Glencross et al., 2005). All extruded diets were made using the same methods and raw materials as used for the screw-press diets. Diets made using extrusion were initially preconditioned with the addition of steam, prior to entry of the mash to the barrel. Barrel temperatures were set at 80, 100 and 140°C from entry to die respectively. Water was also injected into the barrel. A standard salmonid feed screw configuration was used (Evans, 1998). After exit from the die (5mm) the extrudate was cut to produce pellets. The pellets were then dried on a counter-flow heated air drier. Diets were made without the oil component added to the mash. The allotted oil component of each diet was vacuum infused to the pellets following pellet drying.

5.2.2 Fish handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 L). Freshwater (salinity < 1 PSU; Dissolved oxygen 7.0 ± 0.5 mg/L) of 16.0 ± 0.1°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 15 trout of 263.4 ± 45.8 g (mean ± S.D.; n = 40). Treatments were randomly assigned amongst 24 tanks, with each treatment having three replicates.

Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600 each day. The trout were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not contaminated by urine or mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and stored in a freezer at -20°C. Stripped faeces were collected during 0800 to 1000 over a four-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples from different days were pooled within tank, and kept frozen at -20°C before being freeze-dried in preparation for analysis.

5.2.3 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25.

Amino acid analysis involved the samples being hydrolysed at 110°C for 24hr in 6M HCl with 0.05% Phenol. Cysteine and cystine are derivatized during hydrolysis by the addition of 0.05% 3,3'-dithiodipropoinic acid by the method of Barkholt and Jensen (1989). The acid hydrolysis destroyed tryptophan making it unable to be determined. Separation was by HPLC on a Hypersil AA-ODS 5µm column using an 1100 series Hewlett Packard HPLC system. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the Soxhlet method. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

where Y_{diet} and Y_{faeces} represent the chromium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%.

5.2.4 In vivo pellet integrity analysis

At the conclusion of the digestibility study fish from the reference, *L. angustifolius* cv. Myallie kernel meal and soybean meal diet treatments were starved for 24 h. Following this starvation period the fish were fed and three fish culled from each tank (n=3) for each treatment at 2 h, 4 h and 8 h post feeding. The state of the ingested pellets was then examined and given a rank from 0: no loss of integrity, 1: minor sloughing, 2: still distinct pellets through form losing shape, 3: congealed mass of pellets, 4: only large fragments remaining, to 5: complete loss of structural integrity of the pellets. The pellet integrity score for each diet at each time point was calculated as:

$$Score\ (\%) = \frac{5n - (O_0 \times 0 + O_1 \times 1_1 + O_2 \times 2 + O_3 \times 3 + O_4 \times 4 + O_5 \times 5)}{5n} \times 100$$

where n = the number of observations for each treatment (max = 6); O_0 = observed number of samples with a score of 0, O_1= observed number of samples with a score of 1 and so on, and the associated number is the respective score of 0, 1, 2, …5.

5.2.5 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Effects of ingredient on digestibility of dry matter, protein and gross energy in each of the ingredient were examined by one-way ANOVA (Table 5.3). Correlation analysis was performed using Statistic v6. Curve fitting of linear regressed relationships was undertaken using both Microsoft Excel and Statistica v6. Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

5.3 Results

5.3.1 Diet digestibilities

There were several significant differences among the diet digestibility parameters of the extruded and screw-pressed diets (Table 5.3, Figure 5.1). Differences between the diets in terms of their energy digestibility were most distinct, with more significant differences among the test ingredients between the two diet processing methods than that observed for any other parameter. Some significant differences between the diets within test ingredients were also noted for diet digestibilities of dry matter and the sum of amino acids. No significant differences between the diets were noted for diet protein digestibilities.

Correlations between the digestibilities of the extruded and screw-pressed diets were generally high. Dry matter digestibilities of the diets were highly correlated ($R^2 = 0.9545$, $p = 0.0008$). Protein (nitrogen) digestibilities of the diets were highly correlated ($R^2 = 0.9574$, $p = 0.0007$). Energy digestibilities of the diets were most highly correlated of the relationships examined ($R^2 = 0.9973$, $p = 0.0000$). A significant improvement in the digestibility of energy was observed when the diets were extruded. Sum of amino acid digestibilities of the diets were the least correlated of the relationships examined, though still highly significant ($R^2 = 0.8130$, $p = 0.0140$).

5.3.2 Ingredient digestibilities

There were several significant differences among the ingredient digestibility parameters of the extruded and screw-pressed diets (Table 5.3, Figure 5.2). Differences between the ingredients in terms of their energy digestibility were most distinct, with more significant differences among the test ingredients between the two diet processing methods than that observed for any other parameter. Some significant differences between the diets within test ingredients were also noted for ingredient digestibilities of dry matter, protein and the sum of amino acids, most notably soybean meal although the lupin kernel meals also showed some effects of diet processing on ingredient energy digestibilities.

Correlations between the digestibilities of the test ingredients within the extruded and screw-pressed diets were generally high except for those of nitrogen or sum of amino acids. Dry matter digestibilities of the diets were highly correlated ($R^2 = 0.9445$, $p = 0.0056$). Protein (nitrogen)

digestibilities of the ingredients were not well correlated (R^2 = 0.002, p = 0.9429). Energy digestibilities of the ingredients within the diets were most highly correlated of the relationships examined (R^2 = 0.9468, p = 0.0053). The data showed that extrusion of the diets significantly improved the energy digestibility of the test ingredients. Sum of amino acid digestibilities of the test ingredients within the diets were the least correlated of the relationships examined and highly insignificant (R^2 = 0.001, p = 0.9603).

5.4 In vivo pellet integrity analysis

The examination of pellet integrity in the stomach of the fish following feeding showed several significant differences among the three treatments of the fishmeal based reference diet, the lupin (*L. angustifolius* cv. Myallie) kernel meal diet and the soybean meal diet. A significant decline in pellet integrity in the stomach of the fish of both the lupin (*L. angustifolius* cv. Myallie) kernel meal diet and the soybean meal diets was observed at each time point of the study. In contrast no significant changes in the pellet integrity of the reference diet were observed over the 8 h period of the study. Soybean meal had the poorest pellet integrity at all time points of the study and significantly more so than that of he lupin kernel meal. Both the lupin kernel meal and soybean meal diets had poorer pellet integrity at all time points than that of the reference diet.

5.5 Discussion

There have been numerous studies examining the digestible value of feed grains when fed to a variety of fish species (Burel et al., 1998; Booth et al., 2001; Glencross and Hawkins, 2004). Most of these studies have been based on the assessment of the nutritional value of raw materials in diets that have been screw-pressed or at the very least in diets processed using non-commercially applicable processing technology. There have been a few studies examining the impact of pre-extrusion of raw materials on their digestible value or the effect of diet extrusion in general on its nutritional value to fish (Hilton et al., 1981; Allan and Booth, 2004). This study however is the first to examine the digestibility response of a fish to the same diets when processed using either screw-press or extrusion manufacturing technologies. However, an assessment of the effect that these processing technologies have on the assessment of other specific raw materials included as part of the diets for digestibility assessment purposes has not been reported. Presently most laboratory-scale experimental work throughout the world is done using cold-extrusion or screw-press technology. The relevance of feeds processed using this laboratory-scale technology compared to the commercially used steam-injected, heated extrusion equipment has been questioned (Romarheim et al., 2005).

5.5.1 Diet digestibility effects

In this study it was observed that there was a high degree of correlation between the extruded and screw-pressed diets for all digestibility parameters examined. The correlation was proportional in all observed cases, though not necessarily direct in each case. The findings of the present study clearly show that there is a significant benefit of feed extrusion on the energy value of the diets when fed to rainbow trout. This observation is consistent with findings of other researchers that have also reported that extrusion improves the energy digestibility and value of feeds for fish (Hilton et al., 1981; Hilton and Slinger, 1983). It is hypothesised that this is an effect of the extrusion process on the gelatinisation of the starch component of the wheat included in the diet

(Hilton et al., 1981; Bergot and Breque, 1983). Improved nutritional value of gelatinised starch over ungelatinised starch has been previously reported (Jeong et al., 1991).

In contrast no benefit of the extrusion process on the digestible nitrogen or sum of amino acids was observed. This supports that the extrusion process does not have any benefits on the nutritional value of the protein in the diets. In fact in both cases a minor, though not significant, reduction in the protein digestibility was observed between the screw-pressed and extruded diets. This may be attributable to some heat-damage occurring to the protein, but it could also be an artefact of a more strongly bound physical structure resisting the digestive processes more (Glencross et al., 2004c).

5.5.2 Ingredient digestibilities

The findings of the present study also clearly show that there is a significant benefit of diet extrusion on the energy value of the ingredients when fed to rainbow trout. Other researchers examining the pre-extrusion of raw materials prior to incorporation into screw-press made feeds have also reported similar benefits in improved energy digestibilities (Bangoula et al., 1993; Allan and Booth, 2004). In some cases this benefit was explained by the effect of extrusion on the gelatinisation of starch within the raw materials, such as wheat and field peas. However, both soybean and lupins have negligible levels of starch and therefore the reasons for the observed improvement in both the present and the other reported studies are unclear (Bangoula et al 1993).

The lack of a significant correlation between the nitrogen and the sum of amino acid digestibilities between the extruded and screw-pressed diets is interesting. It suggests that the manufacturing process used influences either the nature of the protein in the diet or that there is some other key change in the physical and chemical nature of the diet that is influencing this process. It is probable that there are some interactive effects among the different nutrient classes and compounds in the diets, the way the respond to diet processing. This is clearly an area that requires a more in depth evaluation to determine the specific nature of these interactive effects among nutrient classes.

5.5.3 In vivo pellet integrity

The observations of the in vivo pellet integrity analysis show that raw material choice can have an important role in the physical digestive processes occurring in the stomach of the fish. In this study it was observed that with the addition of either lupin kernel meal or soybean meal to the diet that the rate at which the pellet disintegrated following ingestions was significantly higher than that observed when fishmeal was the only protein source used. Furthermore, there were significant differences between lupins and soybean, in that inclusion of soybean meal produced pellets that disintegrated faster than the pellet with lupin inclusion. Interestingly there were no clear correlations between the measured digestibility parameters of the diets and these physical observations.

Although the specific implications of these physical observations on a nutritional basis remain to be explained. Other studies have identified that the physical durability of pellets can have a significant effect on improving the incidence of fat regurgitation by Pacific salmonids (Baeverfjord et al., 2006). It may be that the harder, more durable physical structure of the lupin pellets compared to the soybean pellets is due in part to the effect that lupin kernel meals have on the pellet binding process during extrusion. It is hypothesised that this is due to an interaction between the starch contributed by the wheat and the other non-starch-polysaccharides contributed by the lupin kernel meal.

5.5.4 Conclusions

The findings of this study confirm that there are both physical and nutritional benefits to aquaculture diets from the extrusion process. The extrusion process specifically improves the digestible energy value of the diets, presumably through the gelatinisation of the starch component of the diets. This effect has also a direct effect on the derived nutritional value of the component test ingredient supporting that extrusion does improve the nutritional value of these feed grains. Similar such improvements in the nutritional value of the overall dry matter or protein components of the diets and ingredients were not observed. These findings show that the strong correlation between the extruded and screw-pressed diets allows for extrapolation of observed digestibility effects from feeds made using either process, although in some cases a conversion factor will be required.

While it is hypothesised that it is the carbohydrates present as in the grains that contribute to much of this variability in nutritional and physical properties the literature so far only details the impacts of starch in this regard. The roles of the complex non-starch polysaccharides on the physical and nutritional properties of the diet and by inference the raw materials remains to explored.

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Table 5.1 Formulations and composition of the extruded (E) and screw pressed (P) experiment diets (all values are g/kg).

	Reference Diet	Soy	APC	LPC	LKM	AKM
Ingredient						
Fishmeal	700.0	490.0	490.0	490.0	490.0	490.0
Fish oil	150.0	105.0	105.0	105.0	105.0	105.0
Soybean meal		300.0				
Angustifolius Protein Concentrate			300.0			
Luteus Protein Concentrate				300.0		
Wodjil kernel meal					300.0	
Myallie kernel meal						300.0
Wheat flour	144.0	100.8	100.8	100.8	100.8	100.8
Vitamin and mineral premix	5.0	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1.0	0.7	0.7	0.7	0.7	0.7
Diet composition as analysed	E/P	E/P	E/P	E/P	E/P	E/P
Dry matter	935/947	938/944	938/942	944/948	925/947	929/942
Protein	514/493	512/508	584/585	593/610	507/522	478/479
Fat	216/234	156/162	190/187	184/174	186/181	177/142
Phosphorus	18/19	15/16	15/15	15/16	15/15	15/14
Ash	124/124	108/109	92/93	93/95	98/101	96/98
Gross Energy	23.5/23.4	22.4/22.3	23.7/23.7	23.6/23.4	23.1/22.7	22.8/22.6

[a] From *L. luteus* (yellow lupins).

[b] From *L. angustifolius* (Sweet lupins).

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Table 5.2 Nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[a] Wheat	Soybean [b]	APC [c]	LPC [d]	AKM [e]	LKM [f]
Dry matter content (g/kg)	931	905	907	926	932	905	909
Crude protein	749	142	521	783	811	425	537
Crude fat	87	24	19	110	55	75	77
Ash	161	11	69	29	32	34	44
Phosphorus	28	2	8	7	8	5	7
Gross energy (MJ/kg DM)	20.5	18.4	19.3	25.1	24.1	20.8	21.1
Arginine	41	7	3.37	7.59	7.33	4.17	5.35
Histidine	13	1	1.30	1.67	1.76	1.08	1.43
Isoleucine	29	5	2.26	3.30	2.90	1.66	2.06
Leucine	56	10	4.06	5.82	6.51	2.91	4.41
Lysine	55	5	2.88	3.32	3.10	1.68	2.71
Methionine	21	2	0.82	0.56	0.62	0.33	0.47
Phenylalanine	30	6	2.72	3.33	3.22	1.76	2.24
Threonine	32	5	2.14	2.54	2.34	1.51	1.92
Valine	33	6	2.13	2.57	2.28	1.40	1.70

[a] Fish meal: Chilean anchovy meal and Australian feed grade wheat, Skretting Australia, Cambridge, TAS, Australia.

[b] Solvent extracted soybean meal (US origin), Wesfeeds, Bentley, WA, Australia.

[c] APC: *L. angustifolius* Protein Concentrate, Department of Agriculture, South Perth, WA, Australia. [d] LPC: *L. luteus* Protein Concentrate, Department of Agriculture, South Perth, WA, Australia. [e]AKM: Sweet lupin: *L. angustifolius* Kernel Meal, WESFEEDS, Bentley, WA, Australia. [f]LKM: Yellow lupin: *L. luteus* Kernel Meal, Coorow Seed Cleaners, Coorow, WA, Australia.

Table 5.3 Digestibility (%) specifications of diets and test ingredients as determined from diets that were processed using either extrusion or screw-press technologies.

	Reference	Soybean	APC	LPC	AKM	LKM	Pooled EM
Diet Digestibility - Extruded							
Dry matter	$^{a}0.818^{ab}$	$^{a}0.765^{b}$	$^{a}0.847^{a}$	$^{a}0.837^{a}$	$^{a}0.733^{c}$	$^{a}0.778^{b}$	0.0071
Energy	$^{a}0.910^{a}$	$^{a}0.850^{bc}$	$^{a}0.913^{a}$	$^{a}0.906^{a}$	$^{a}0.831^{c}$	$^{a}0.862^{b}$	0.0064
Protein	$^{a}0.899^{b}$	$^{a}0.901^{b}$	$^{a}0.925^{a}$	$^{a}0.921^{a}$	$^{a}0.899^{b}$	$^{a}0.906^{ab}$	0.0020
Sum Amino Acids	$^{a}0.928^{ab}$	$^{a}0.917^{b}$	$^{a}0.942^{a}$	$^{a}0.935^{a}$	$^{a}0.924^{b}$	$^{a}0.931^{ab}$	0.0013
Ingredient Digestibility- Extruded							
Dry matter		$^{a}0.645^{b}$	$^{a}0.907^{a}$	$^{a}0.885^{a}$	$^{a}0.493^{c}$	$^{a}0.650^{b}$	0.0234
Energy		$^{a}0.705^{c}$	$^{a}0.888^{a}$	$^{a}0.902^{a}$	$^{a}0.696^{c}$	$^{a}0.785^{b}$	0.0170
Protein		$^{a}0.970^{a}$	$^{a}0.975^{a}$	$^{a}0.965^{a}$	$^{a}0.922^{b}$	$^{a}0.925^{b}$	0.0057
Sum Amino Acids		$^{a}0.703^{c}$	$^{a}0.993^{a}$	$^{a}0.756^{c}$	$^{a}0.910^{b}$	$^{a}1.074^{a}$	0.0240
Diet Digestibility – Pressed							
Dry matter	$^{a}0.825^{a}$	$^{b}0.736^{c}$	$^{a}0.834^{a}$	$^{a}0.827^{a}$	$^{a}0.722^{c}$	$^{a}0.765^{b}$	0.0071
Energy	$^{b}0.889^{a}$	$^{b}0.820^{b}$	$^{b}0.894^{a}$	$^{b}0.880^{a}$	$^{b}0.798^{c}$	$^{b}0.835^{b}$	0.0064
Protein	$^{a}0.903^{ab}$	$^{a}0.897^{b}$	$^{a}0.928^{a}$	$^{a}0.925^{a}$	$^{a}0.900^{ab}$	$^{a}0.908^{a}$	0.0020
Sum Amino Acids	$^{a}0.933^{ab}$	$^{a}0.926^{b}$	$^{a}0.949^{a}$	$^{b}0.949^{a}$	$^{a}0.937^{a}$	$^{a}0.936^{a}$	0.0013
Ingredient Digestibility - Pressed							
Dry matter		$^{b}0.543^{a}$	$^{a}0.860^{c}$	$^{a}0.848^{c}$	$^{a}0.489^{a}$	$^{a}0.651^{b}$	0.0234
Energy		$^{b}0.628^{b}$	$^{a}0.880^{d}$	$^{a}0.834^{d}$	$^{a}0.558^{a}$	$^{a}0.692^{c}$	0.0170
Protein		$^{b}0.920^{a}$	$^{a}0.980^{b}$	$^{a}1.033^{b}$	$^{a}0.936^{a}$	$^{a}1.008^{b}$	0.0057
Sum Amino Acids		$^{b}1.082^{a}$	$^{a}1.064^{a}$	$^{a}1.139^{a}$	$^{a}1.265^{b}$	$^{a}1.100^{a}$	0.0240

Different pre- superscripts within columns indicate significant differences between means among the same digestibility parameters between diet types, but not between different digestibility parameters or between different ingredients ($P < 0.05$).

Different post- superscripts within rows indicate significant differences between means among ingredients, but not between digestibility parameters or diet type ($P < 0.05$).

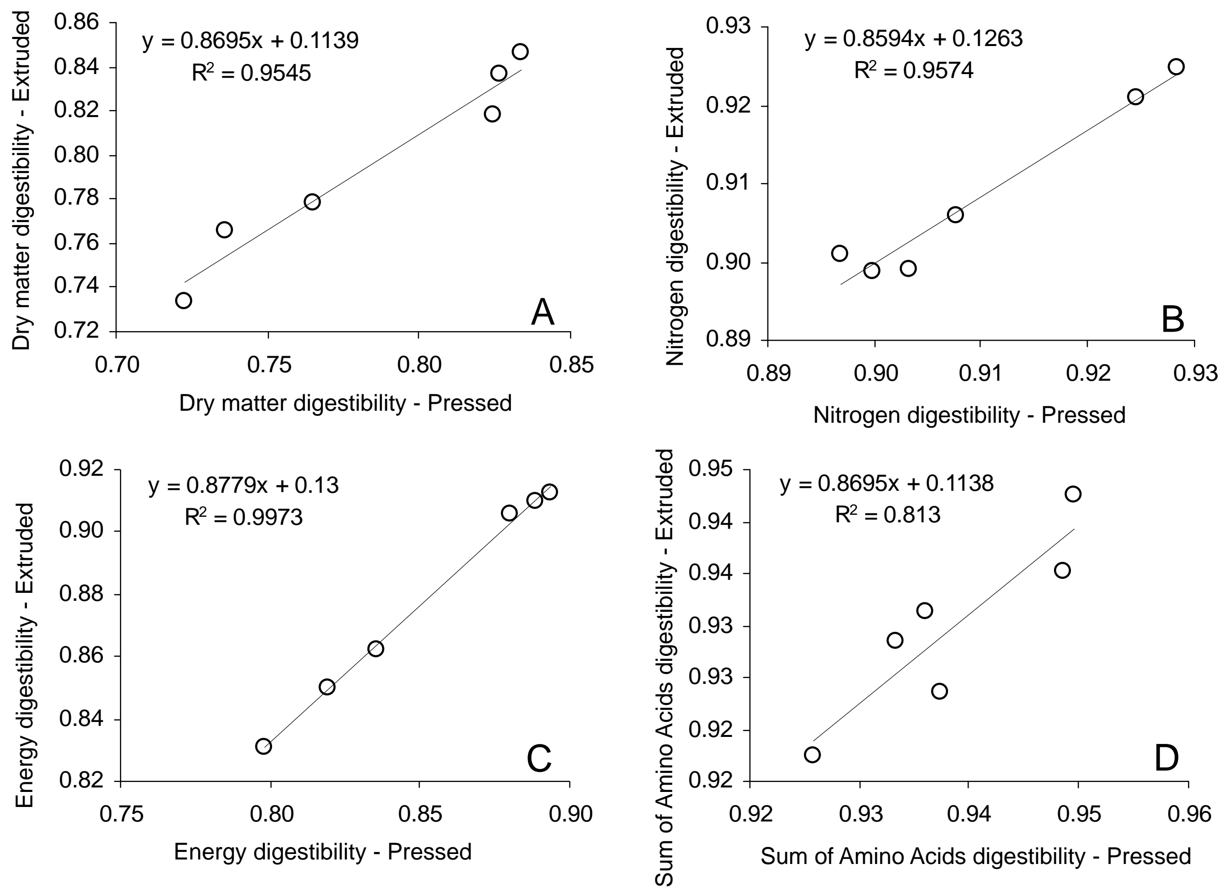

Figure 5.1 Correlations among diet digestibilities of the same formulations when either extruded or pressed. Shown are the dry matter (A), nitrogen (B), energy (C), and sum of amino acid (D) digestibilities.

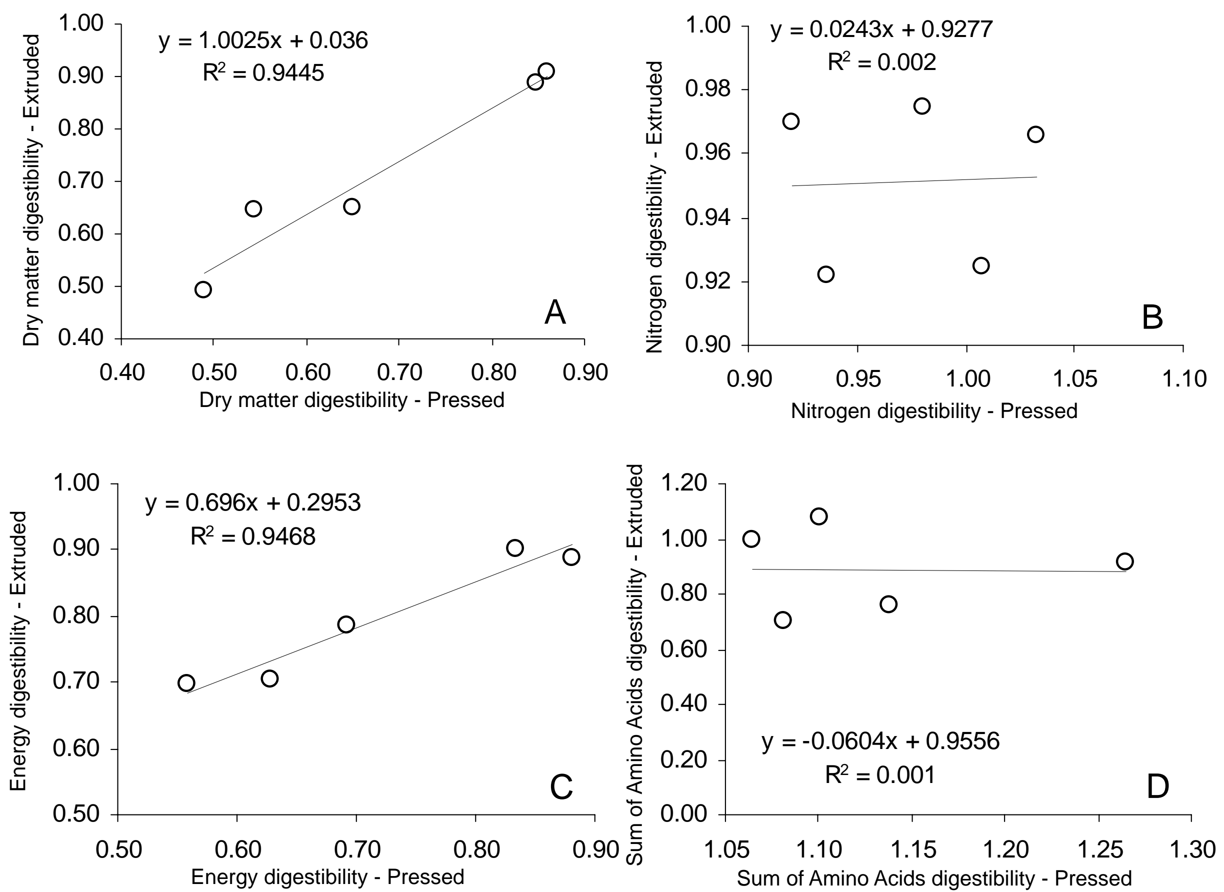

Figure 5.2 Correlations among ingredient digestibilities of the same formulations when either extruded or pressed. Shown are the dry matter (A), nitrogen (B), energy (C), and sum of amino acid (D) digestibilities of each of the test ingredients.

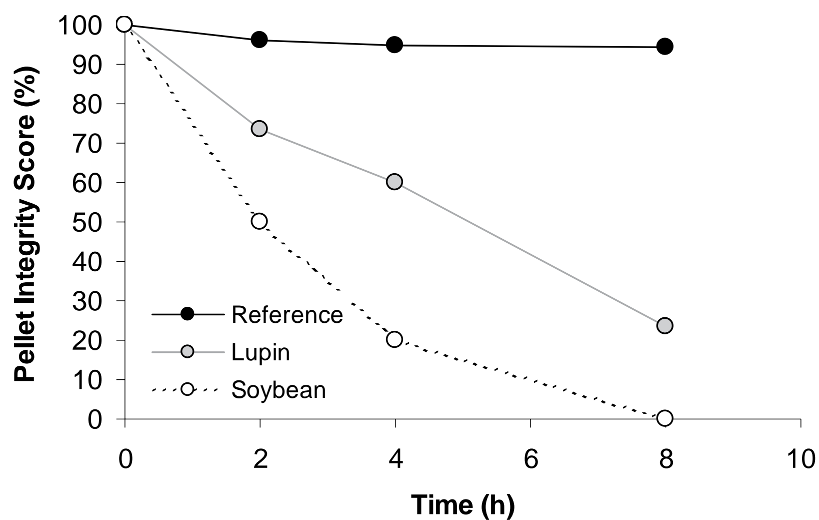

Figure 5.3 *In vivo* pellet integrity scores of different extruded diets fed to rainbow trout at various time points post-feeding. Notable is the significant deterioration in pellet integrity of the two grain test diets and that soybean meal resulted in poorer pellet integrity in the fish's stomach at every time point of the study.

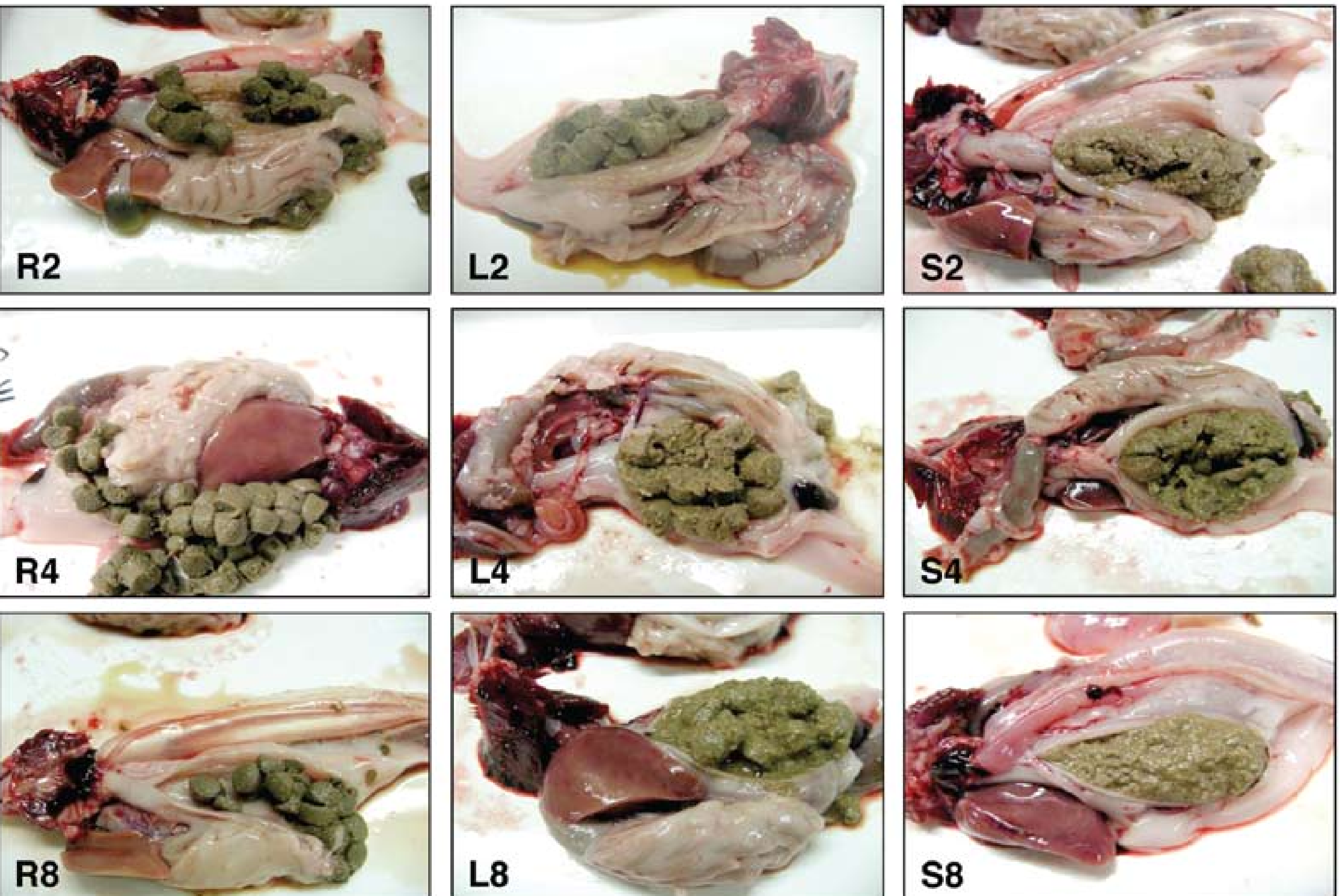

Figure 5.4 Photographs of the stomach contents of fish from each diet treatment at 2 h, 4 h and 8 h to provide an indicative scale of the loss of pellet integrity over the period of the study. Figure R1 was scored 0 and Figure S8 was scored 5. Figures L4 and S4 were scored 2 and 3 respectively. R: Reference diet, L: Lupin (*L. angustifolius*) kernel meal diet, S: Soybean meal diet. Number refers to hour post feeding (e.g. R4 = reference diet 4 h post feeding).

6.0 Assessing the variability in the chemical and physical characteristics of lupin (*Lupinus angustifolius*) kernel products

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Abstract

This study examined the variability in chemical composition and physical hardness of lupin kernels. Seventy-five samples of lupins were collected over a three-year period. Of those 75 samples, 39 samples constituted repeated samples of the same genotype grown at the same location in three successive years. Each of the lupin samples was dehulled and a lupin meal/ flour produced.

Mean protein across all samples was 45.4% on a dry basis. Protein based on sum of amino acids was marginally lower at 44.0% and an improved transformation factor for nitrogen to protein based on N x 6.02, for lupins is proposed. Total lipid was 7.8% and ash 3.0%. Carbohydrates, measured by difference between dry matter minus protein, lipid and ash, were 43.8% on a dry basis. Mean gross energy was 20.8 MJ/kg DM. Protein ranged from 36.5% to 56.7% with a coefficient of variation (CV) about the mean of 7.6%. Variation in fat/lipid levels was greater with a CV of 12.1%. Gross energy ranged from 20.1 to 21.5 MJ/kg DM with a CV of 15.3%. Substantial variability was also observed in the amino acid composition of the samples, with some amino acid CV's up to 32%.

Significant variance was observed between years across the 15 commercial cultivars (genotype) grown at the same site in successive years (2002, 2003 and 2004). Variance as a function of growing year was greater than that attributable to genotype.

Lupin kernel hardness, as assessed by cutting of a kernel by a texture meter, was assessed based on the overall force required to split a kernel and also the rate of force application. The later representing whether a grain cracked or tore. The high force required to split some grain varieties was consistent with these varieties being easier to mill. The rate of force application was even more consistent with anecdotal evidence on milling ease, with lower rates of force application consistent with greater difficulty in milling. Care should be taken when broadly applying findings from the texture meter work, though, as discrepancies are likely to exist depending on grain variety.

6.1 Introduction

Variability is inherent in all raw materials. Understanding the nature and extent of this variability is the first step towards its management. Typically, this variability is managed, to an extent, by the bulk blending of a range of grain stocks to create a larger homogenous pool of grain. This has certainly been the primary mode of quality management for lupins. However, in doing so the higher value, higher quality grain is diluted. Because grain value is not necessarily linear with regards to its protein content, this represents a significant loss in value (Kingwell, 2003).

Greater value could be captured by segregating the lupins into high-protein and low-protein pools, with the high-protein lupins being the primary raw material for further value-adding, while the low-protein lupins would be adequate for the ruminant market, where the value is based primarily on their metabolisable energy value, not the digestible protein value (Edwards and Van Barneveld, 1998). While some studies have been undertaken to examine potential agricultural factors that affect chemical quality traits in lupins, no definable criteria have proven to be reliable in predicting these traits consistently. While genotype has been touted as one avenue to manage quality traits like crude protein content, agricultural region was shown to have some significant influence. It was suggested that the drier regions consistently produced lupins with higher protein concentrations (Cowling and Tarr, 2004; French, 2005). Notably Lupin-Zones 3 and 7 consistently produce higher-protein lupins than the other regions (Figure 6.1).

In addition to their chemical composition, the physical hardness of a lupin kernel also has important ramifications in regard to the energy demand and potential throughput in milling of the kernels (Sipsas et al., 2005). Anecdotal information had indicated that lupins were considered hard to mill and that this was a potential bottleneck in their use in aquaculture feed mills, where all raw materials are much more finely ground than that required in feeds for other species.

This study reports on the variation in composition of a collection of 75 narrow-leaf lupin, (*Lupinus angustifolius*) samples when they have been processed to a kernel meal, with a focus on the composition of certain genotypes from successive years grown at the same site. In addition, the physical characteristics of the lupin kernels from a range of cultivars is also examined to consider the variability in the force required to cleave the kernels, which is indicative of their hardness in milling.

6.2. Materials and Methods

6.2.1 Ingredient and diet development

Over a three-year period, separate batches of seed of *Lupinus angustifolius* were collected from the Department of Agriculture's (WA) germplasm and breeding lines. This seed in many cases constituted the same genotype over several seasons, often from the same site (Wongan Hills Research Station; Latitude S 38°.84', Longitude E 116°.73', Altitude 305 m). Samples of the seed were then split using a small disc-mill and aspirated to separate hulls from kernels. A final manual clean of the kernels to remove any remaining hull material was also undertaken on each sample to ensure purity of the kernel preparation. Sub-samples were kept of each kernel preparation. The remainder of each kernel sample was then milled using a Restsch Hammermill with a 750 µm screen to create a kernel flour.

6.2.2 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Lupin kernel meal samples were analysed for dry matter, total lipids, ash, phosphorus, nitrogen, amino acids and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Amino acid analysis involved the samples being hydrolysed at 110°C for 24hr in 6M HCl with 0.05% Phenol. Cysteine and cystine

are derivatized during hydrolysis by the addition of 0.05% 3,3'-dithiodipropionic acid. The acid hydrolysis destroyed tryptophan making it unable to be determined. Separation was by HPLC on a Hypersil AA-ODS 5µm column using an 1100 series Hewlett Packard HPLC system. Total lipid content of the kernel meals was determined gravimetrically following extraction of the lipids according to the method of Folch et al. (1957). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry.

6.2.3 Kernel hardness/ Shear strength

The hardness of the lupin kernels from each culitvar was assessed based on the force to shear a cotyledon/kernel across their lateral diameter. The assessment was made using a Stable Microsystems TA-XT2 texture meter (Arrow Scientific, Leichhardt, Australia) with a 15,000 g load-cell and a utility knife blade as the cutting edge. Seven kernels from each treatment were assessed for their hardness. The force to shear the kernels was measured as grams of pressure as compression. The texture analyser was set with a pre-test speed of 2 mm/s with a test speed of 0.1 mm/s. The blade was set to pass a maximum distance of 2 mm and trigger at a contact pressure of 10 g. Shear strength was defined as the peak force at breaking of the kernel.

6.2.4 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Comparisons of means of individual compositional parameters were examined by one-way ANOVA , followed by a LSD planned comparisons post-hoc test. Limits for all critical ranges were set at $P < 0.05$.

6.3 Results

6.3.1 Variability in composition

The (mean ± S.D.) protein concentration in lupin kernels, across all 75 samples, was 45.4 ± 3.4% on a dry basis. Total lipid was 7.8 ± 0.9% and ash 3.0 ± 0.4%. Carbohydrates, measured by difference between dry matter minus protein, lipid and ash, were 43.8 ± 3.3% on a dry basis. Mean gross energy was 20.8 ± 0.3 MJ/kg DM (Table 6.1; Figure 6.2).

There was substantial variation in most compositional parameters. Lupin kernel protein ranged from 36.5% to 56.7% with a coefficient of variation (CV) about the mean of 7.6%. Variation in fat/lipid levels was greater with a CV of 12.1%. Variation in carbohydrate levels was greater still with a CV of 13.0%. Gross energy ranged from 20.1 to 21.5 MJ/kg DM and had a CV of 15.3%. Substantial variability was also observed in the amino acid composition of the samples, with some amino acid CV's up to 32% (Table 6.1). Methionine was a notable example in that its concentration ranged from 0.2% to 0.7% with a CV about the mean of 32.2%. An inverse logarithmic relationship was noted between mean amino acid concentration and the CV.

6.3.2 Genotype effects on composition

Significant variance was observed between the 15 commercial cultivars grown at the same site (Table 6.2). Comparisons with-in years showed that the varieties Coromup and Gungurru had consistently the highest crude protein, while Belara consistently had the lowest crude protein concentrations. When protein was assessed as the sum of amino acids Gungurru had clearly the

highest protein levels and Mandelup and Belara the lowest. There was some discrepancy between the protein estimation methods based on nitrogen x 6.25 or the sum of amino acids. In most cases the sum of amino acids provided a more conservative estimate. Total lipid concentrations were highest in the Belara cultivar and lowest in the Moonah cultivar. Gross energy levels were highest in Danja, reflecting the high levels of both protein and lipid in that cultivar. Gross energy levels were lowest in the Moonah and Tallerack cultivars, consistent most with the low total lipid levels in these cultivars.

6.3.3 Season/Year effects on composition

Significant variance was observed between years across the 15 commercial cultivars grown at the same site in successive years (2002, 2003 and 2004) (Figure 6.2). Variance as a function of growing year was greater than that attributable to genotype (Figure 6.3). Protein concentration of the kernel was greatest from the 2003 season and lowest from the 2002 season. Total lipids content did not show as much variation as protein, but was highest in samples from the 2002 season and lowest from the 2003 season. Gross energy content of the kernels was highest from the 2004 season, while energy content from either the 2002 or 2003 seasons were similar.

6.3.4 Kernel hardness

Lupin kernel hardness, as assessed by cutting of individual kernels by a texture meter, was assessed based on both the overall force required to split a kernel and also the rate of force application. Substantial variability in the peak force required to split kernels was observed between the different *L. angustifolius* cultivars (Figure 6.4, 6.6 and 6.7). A high level of variability was also observed between the same varieties but from different seasons (Figure 6.7). Similar levels of variability were also observed between *L. angustifolius* and *L. luteus* (Figure 6.5 and 6.6). Kernels of *L. albus* were shown to be significantly harder than those of either *L. angustifolius* or *L. luteus* (Figure 6.7). The *L. albus* cv. Kiev mutant was the hardest of the lupin varieties, while *L. angustifolius* varieties of Kalya, Merrit, Mandelup and Tanjil were among the softest. Belara was the hardest of the *L. angustifolius* varieties.

The rate of force application showed less variability overall, but an improved discernability among the different varieties of *L. angustifolius*. The *L. angustifolius* cv Belara had the slowest force rate, while Quilinock had the fastest of the lupin kernels.

6.4 Discussion

6.4.1 Variability in lupin kernel composition

The mean protein concentration in the lupin kernels, across all 75 samples of 45.4 ± 3.4% was consistent with most other recently published studies on *L. angustifolius* kernel meals (Glencross et al., 2004; 2005; 2007). In comparison to earlier published works, many of the more recent evaluations of kernel meals have marginally higher protein content (Petterson et al., 1997; Edwards and Van Barneveld, 1998). Based on the lupin varieties assessed in this study it is likely that this difference in protein concentration is not a genotype effect (especially as many of the newer released cultivars; Mandelup, Belara, Tanjil, are lower protein varieties), but probably a processing effect. The more recent processing of lupins being a more efficient process producing a cleaner kernel preparation with reduced hull content and therefore a higher protein concentration. The assessment of carbohydrates, as measured by difference between dry matter minus protein, lipid and ash, shows that this parameter is largely a reciprocal of the total protein

content, as the lipid and ash concentrations are low and not that variable (Table 6.1). Mean gross energy across all samples was 20.8 ± 0.3 MJ/kg DM (Table 6.1; Figure 6.2). Based on energetic values of 23.6 kJ/g, 38.5 kJ/g and 17.3 kJ/g for protein, lipid and carbohydrate respectively the mean composition estimate is closer to 21.3 MJ/kg DM (AOAC, 2005). The actual energy values presented, though, are based on calorimetric measurements, not calculations. Therefore this discrepancy perhaps indicates that the protein level estimated based on N x 6.25 may not be accurate. Assessment of energy based on the same assumptions, but using the sum of amino acids as a protein estimate yields 20.9 MJ/kg DM as an energy value, substantially closer to the actual value measured. Although the standard transformation factor for nitrogen to protein is x 6.25 (AOAC, 2005), based on the sum of the amino acids by the nitrogen content from the 75 samples a transformation factor for nitrogen to protein of N x 6.02 ± 0.168% would be more appropriate.

Among most compositional parameters there was substantial variation. Protein concentrations ranged from 36.5% to 56.7% with a coefficient of variation (CV) about the mean of 7.6%. If a standard commercial kernel meal of 38% protein (42% dry basis) achieves a market value of AUD$350 /tonne f.o.b., then a 56% protein kernel meal has a value of AUD$464 /tonne f.o.b. Even if through segregation two grades are achieved (e.g. < 45% or > 45% dry basis) then the resultant average protein levels based on the data obtained in this set of 75 samples would produce kernel meals with protein concentrations of 42.8% and 48.0% (dry basis) respectively. These kernel meals would have a value of AUD$355 /tonne f.o.b. and AUD$398 /tonne f.o.b. respectively. Although this assessment does make the assumption that the 75 lupin sample in this study are a representative sample of that produced in the grain production region and that the kernel protein concentrations achieved in this study are achievable commercially (which are not necessarily valid in this case), it does show the potential gains achievable through a segregation process. The assumption also assumes that every % protein is worth AUD$8.29 irrespective of protein level, which is also not a valid assumption as the relationship between value and protein is not necessarily linear one, but more likely to be an exponential one (Kingwell, 2003).

The substantial variability observed in the amino acid composition of the samples, with some amino acid CV's up to 32% is probably due to the higher level of error associated with the analysis of these parameters and in particular the less abundant amino acids (Table 6.1). Methionine was a notable example in that its concentration ranged from 0.2% to 0.7% with a CV about the mean of 32.2%. Supporting this premise of the influence of sample concentration on the variability in the assessment of each parameter, an inverse logarithmic relationship was noted between mean amino acid concentration and the CV.

6.4.2 Influence of genotype on composition and hardness

The evaluation of the variation between the 15 commercial cultivars grown at the same site (Table 6.2) showed that there were significant differences between the different cultivars/genotypes. For a valid comparison, the comparisons were made only within years and from grain grown at the same site (Wongan Hills Research Station). These comparisons showed that the varieties Coromup and Gungurru had consistently the highest crude protein, while Belara consistently had the lowest crude protein concentrations. Notably, Coromup is the latest cultivar release by the Western Australian Government Department of Agriculture and Food. Its release promoted this feature as a highlight of the variety. In contrast, cultivars like Belara, Tanjil and Mandelup, while high yielding have been recognised as low protein varieties. Because there is some genotype effect on protein there may be value in using this as means of increasing the protein content of specific segregations. However, the gains that may be made in promoting further increases in protein content of *L. angustifolius* are still not

likely to be as great as that achieved by improving production characteristics of the higher protein lupin *L. luteus*.

Substantial variability in the peak force required to split kernels was observed between different *L. angustifolius* cultivars. This lupin kernel hardness, as assessed by the cutting of individual kernels by a texture meter, measured both the overall force required to split a kernel and also the rate of force application. Similar levels of variability were also observed between *L. angustifolius* and *L. luteus*. Kernels of *L. albus* were shown to be significantly harder than those of either *L. angustifolius* or *L. luteus*. The *L. albus* cv. Kiev mutant was the hardest of the lupin varieties, while *L. angustifolius* varieties of Kalya, Merrit, Mandelup and Tanjil were among the softest. Belara was the hardest of the *L. angustifolius* varieties. Anecdotally, *L. albus* is reputedly an easy lupin variety to mill and therefore it reasons that a "hard" lupin shatters easier and is therefore easier to mill, while a "soft" lupin, like Belara, doesn't shatter, but rather tears and shreds and is not effectively milled in percussion milling systems like a hammer mill. The factors that affect this milling ability of lupins need to be more fully investigated. Factors such as composition (moisture, protein, fat, different carbohydrate classes) and storage time and interactions between these factors are suggested as possible things that may affect the milling quality of lupins.

The rate of force application showed less variability overall, but an improved discernability among the different varieties of *L. angustifolius*. The *L. angustifolius* cv Belara had the slowest force rate, while Quilinock had the fastest of the lupin kernels. This slowest force rate also perhaps being consistent with the hypothesis of a tearing plant structure rather than a shattering one.

6.4.3 Influence of season on composition and hardness

Significant variance was observed between each of the three sample years (2002, 2003 and 2004) across each of the 15 commercial cultivars grown at the same site (Figure 6.2). Based on the variance in key compositional parameters, the variability as a function of growing year was generally greater than that attributable to genotype (Figure 6.3). This is consistent with reports by other studies that have suggested that the most reliable mechanism to separate lupins based on protein content was by Lupin-Zone (Figure 6.1). In this context, the higher protein levels were observed from the drier cropping areas (Cowling and Tarr, 2004; French, 2005). However, these authors also proposed that the main mechanism available to farmers to improve the protein content of their grain was through cultivar choice.

Across the three years evaluated, the protein concentration of the kernel was highest from the 2003 season and lowest from the 2002 season. Total lipids content did not show as much variation as protein, but was highest in samples from the 2002 season and lowest from the 2003 season. Gross energy content of the kernels was highest from the 2004 season, while energy content from either the 2002 or 2003 seasons were similar (Figure 6.2). Based on growing season climates in these three years it appears that protein is higher in years of greater rainfall and lower in periods of drought (ABARE, 2006). This observation contrasts with the observation of higher protein levels in seeds from Lupin-Zones 3 and 7, which are drier lupin production zones (Cowling and Tarr, 2004; French, 2005).

Some variability in the hardness of the lupin kernels from the Belara cultivar was also observed (Figures 6.7 and 6.8). The influence of environmental factors (site and season) on physical parameters such as hardness needs to be more fully followed up.

6.4.4 Conclusion

Earlier works have examined in detail the effects of genotype and environment on the composition of whole lupins, but not lupin kernels (Cowling and Tarr, 2004). The findings in this study show that both genotype and environment have a significant effect on the composition variability in lupin kernels. However, with only three years worth of data and only one site considered, further work is required on this topic to formulate more robust conclusions.

The work on the hardness of the lupin kernels needs further validation. Ideally hammer milling of kernel samples with a recording on energy demand (kW), time of throughput or other such functional parameters needs to be undertaken to allow the development of more meaningful assessments from equipment like the texture meter. Accordingly, care should be taken when applying findings from the texture meter work, as discrepancies are likely to exist depending on grain variety and the interpretation of this data.

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Tables and Figures

Table 6.1 Mean composition (% dry basis unless otherwise detailed) parameters across all (n=75) lupin (*L. angustifolius*) kernel meals samples from the study set.

	Mean	SD	CV%	Minimum	Maximum
Dry matter (% as is)	91.6	0.6	0.6%	90.4	92.8
Protein	45.4	3.4	7.6%	36.5	56.7
Fat	7.8	0.9	12.1%	5.2	9.7
Ash	3.0	0.4	5.9%	1.9	3.9
Carbohydrate	43.8	3.3	14.0%	32.7	53.9
Dietary Fibre*	30.9	4.6	14.9%	17.5	43.4
Acid Detergent Fibre*	6.6	4.5	69.1%	3.0	20.0
Neutral Detergent Fibre*	10.2	5.4	52.3%	5.2	26.2
Lignin*	0.6	0.4	57.6%	0.2	2.2
Phosphorus	0.4	0.1	7.6%	0.3	0.6
Energy (MJ/kg dry basis)	20.8	0.3	15.3%	20.1	21.5
Nitrogen	7.3	0.6	7.6%	5.8	9.1
Sum of Amino Acids	44.0	3.2	7.2%	33.2	53.7
Alanine	1.6	0.1	6.8%	1.3	1.8
Arginine	5.1	0.5	9.9%	4.0	6.6
Aspargine	4.9	0.4	7.7%	3.8	5.9
Cysteine	0.7	0.1	16.5%	0.5	1.3
Glutamate	10.0	0.8	7.8%	7.5	12.6
Glycine	1.9	0.1	6.4%	1.5	2.1
Histidine	1.1	0.1	11.8%	0.8	1.4
Isoleucine	1.7	0.1	7.6%	1.3	2.0
Leucine	3.2	0.3	8.0%	2.4	4.3
Lysine	1.8	0.2	13.2%	1.2	2.4
Methionine	0.3	0.1	32.2%	0.2	0.7
Phenylalanine	1.8	0.2	12.4%	0.1	2.1
Proline	2.5	0.6	26.0%	1.0	4.3
Serine	2.4	0.2	6.8%	1.9	2.9
Threonine	1.8	0.1	7.3%	1.5	2.1
Tyrosine	1.7	0.2	9.1%	1.1	2.1
Valine	1.5	0.1	8.4%	1.2	1.8

CV% is Coefficient of Variation (SD / Mean) x 100

Table 6.2 Mean composition of kernel meals of commercial *L. angustifolius* cultivar genotypes sourced from Wongan Hills Research Station from seasons 2002, 2003 and 2004. All values are % dry basis unless otherwise detailed.

	Belara	Coromup	Danja	Gungurru	Jindalee	Kalya	Mandelup	Merrit	Moonah	Myallie	Quilinock	Tallerack	Tanjil	Wonga	Yorrel	Pooled SEM
Dry matter (% as is)	92.0	91.9	91.4	91.7	92.4	91.8	91.7	92.3	92.5	92.1	91.8	91.8	91.9	92.2	91.9	0.08
Protein	44.9	47.7	47.8	48.0	46.4	47.3	45.9	47.5	47.1	45.8	46.5	47.0	46.8	45.7	45.1	0.50
Total lipid	8.5	7.8	7.9	7.2	8.3	7.2	7.4	7.0	6.9	7.5	7.4	7.0	8.0	7.4	7.6	0.15
Ash	2.8	2.8	3.0	3.2	2.8	3.1	2.9	3.1	2.8	2.9	2.9	3.0	2.9	3.1	2.7	0.08
Carbohydrate	43.8	41.8	41.2	41.7	42.5	42.3	43.8	42.3	43.2	43.8	43.2	43.0	42.4	43.8	44.6	0.46
P	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.4	0.5	0.4	0.4	0.3	0.01
Energy (MJ/kg DM)	20.8	20.8	21.0	20.7	20.7	20.8	20.8	20.7	20.6	20.7	20.7	20.6	20.9	20.7	20.8	0.05
N	7.2	7.6	7.6	7.7	7.4	7.6	7.3	7.6	7.5	7.3	7.4	7.5	7.5	7.3	7.2	0.08
Sum of Amino Acids	42.3	45.4	45.9	48.4	42.9	47.1	42.8	47.0	45.9	45.5	43.9	43.3	45.1	44.7	43.8	0.44
Alanine	1.5	1.7	1.7	1.6	1.5	1.6	1.6	1.6	1.6	1.6	1.5	1.5	1.6	1.5	1.5	0.02
Arginine	4.7	5.4	5.2	5.9	5.0	5.5	4.7	5.7	5.3	5.3	4.9	4.9	5.3	5.3	5.0	0.08
Asparagine	4.5	5.0	5.2	5.2	4.8	5.2	4.7	5.1	5.1	5.2	4.9	4.8	5.0	4.9	4.8	0.06
Cysteine	0.7	0.6	0.7	0.7	0.7	0.8	0.7	0.6	0.7	0.6	0.7	0.6	0.7	0.7	0.7	0.01
Glutamine	9.2	10.0	10.3	11.1	10.0	10.7	9.5	10.7	10.3	10.3	10.0	9.7	10.1	10.3	9.8	0.11
Glycine	1.8	1.9	2.0	1.9	1.7	1.9	1.8	1.9	1.9	1.9	1.8	1.8	1.9	1.8	1.8	0.02
Histidine	1.0	1.1	1.2	1.1	1.0	1.1	1.1	1.1	1.1	1.1	1.0	1.0	1.1	1.0	1.0	0.02
Isoleucine	1.6	1.7	1.8	1.8	1.7	1.8	1.7	1.8	1.8	1.8	1.7	1.7	1.8	1.7	1.6	0.02
Leucine	3.1	3.3	3.4	3.4	3.1	3.4	3.1	3.3	3.3	3.3	3.2	3.1	3.2	3.2	3.2	0.03
Lysine	1.9	2.0	2.0	2.0	1.8	2.0	1.7	1.9	1.9	1.8	1.8	1.9	1.9	2.1	2.0	0.04
Methionine	0.3	0.3	0.2	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.01
Phenylalanine	1.7	1.8	1.8	2.0	1.7	1.9	1.8	1.9	1.9	1.9	1.8	1.8	1.8	1.8	1.8	0.01
Proline	3.0	2.8	2.5	3.4	2.5	3.1	2.7	3.3	2.9	2.8	2.8	2.7	3.0	2.6	2.8	0.07
Serine	2.3	2.4	2.5	2.6	2.4	2.6	2.3	2.5	2.4	2.5	2.4	2.4	2.4	2.4	2.3	0.02
Threonine	1.7	1.9	1.9	1.7	1.6	1.8	1.7	1.7	1.8	1.7	1.7	1.7	1.8	1.6	1.7	0.02
Tyrosine	1.7	1.8	1.9	1.9	1.8	1.8	1.7	1.9	1.9	1.9	1.8	1.7	1.8	1.7	1.7	0.02
Valine	1.5	1.6	1.6	1.6	1.5	1.5	1.6	1.6	1.6	1.5	1.5	1.5	1.5	1.5	1.5	0.02

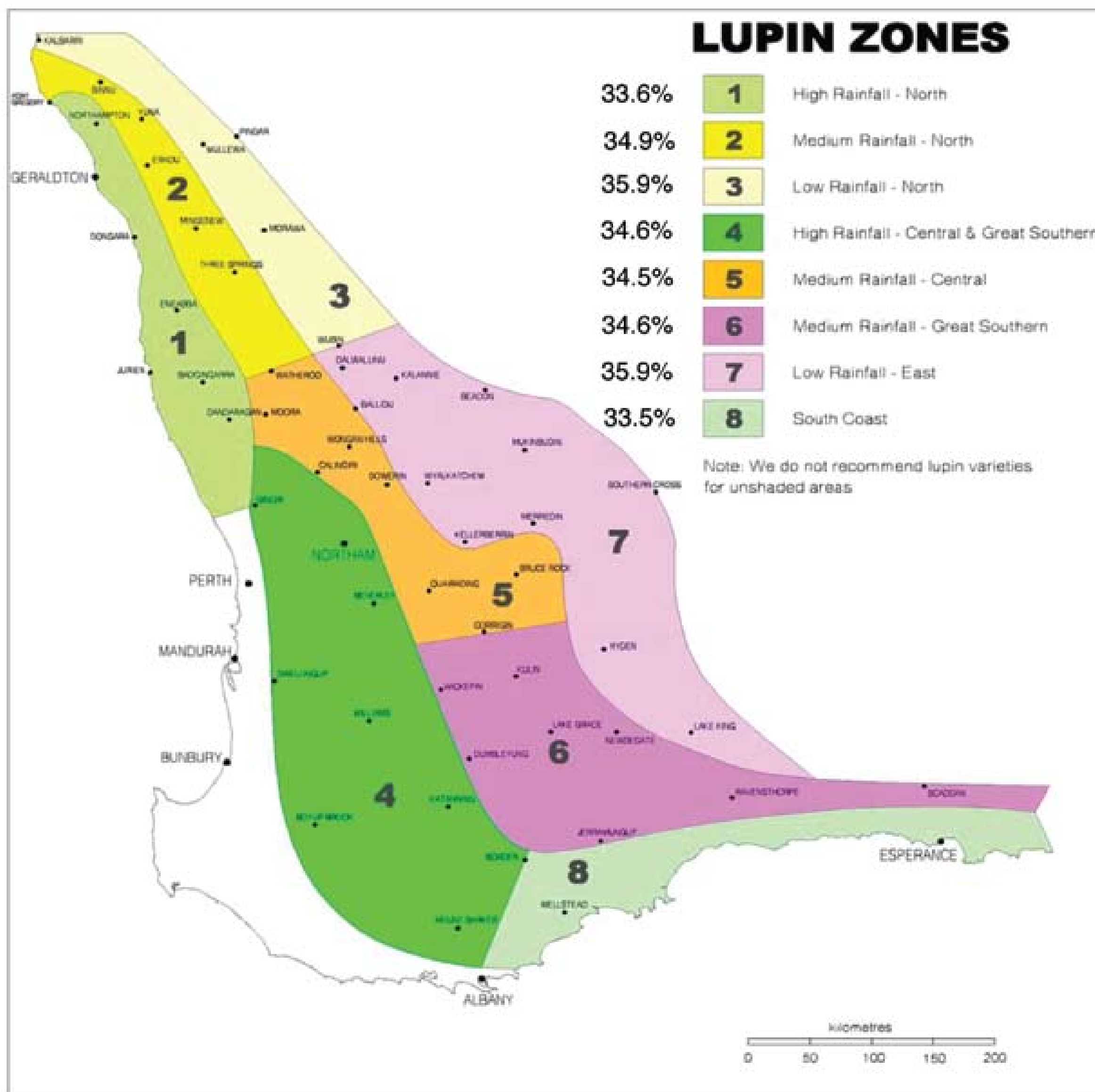

Figure 6.1 Lupin-Zones of the Western Australian grain production region. Each Lupin-Zone is characterised according to both climatic and geographic features. The mean protein content of whole-seed lupin within each Lupin-Zone, on a dry-basis is detailed next to the legend.

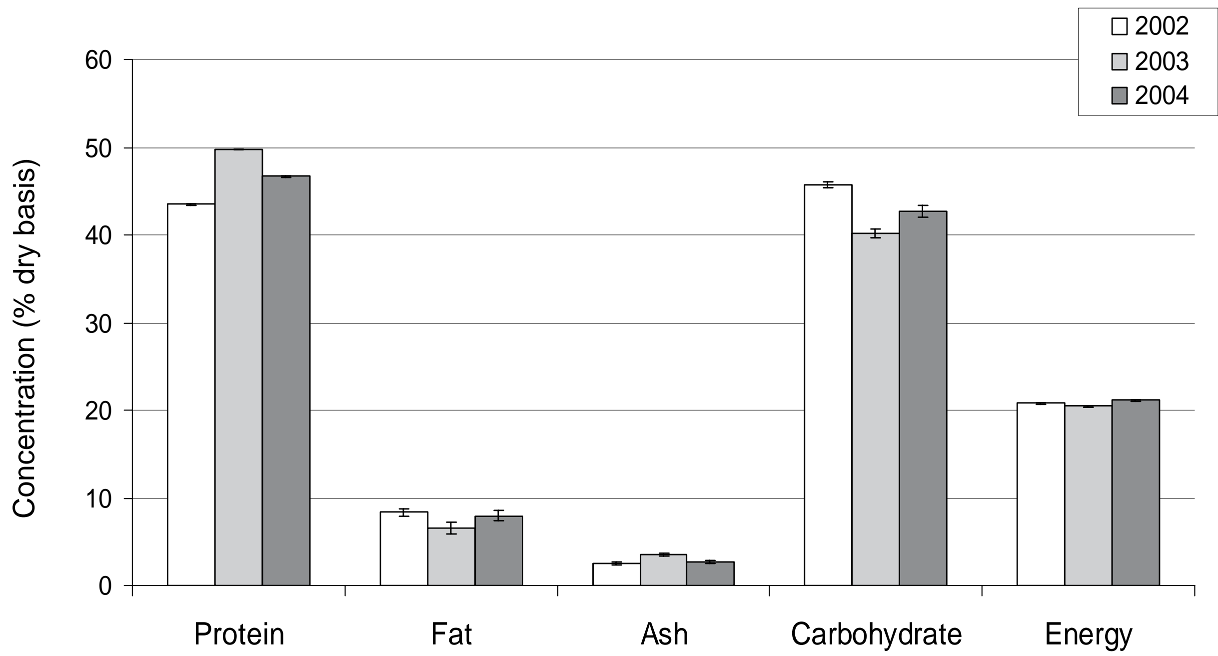

Figure 6.2 Variation in composition of lupin kernel meals of 15 commercial cultivars across three years, all grown at the same site (Wongan Hills, Western Australia). Values are means ± SEM.

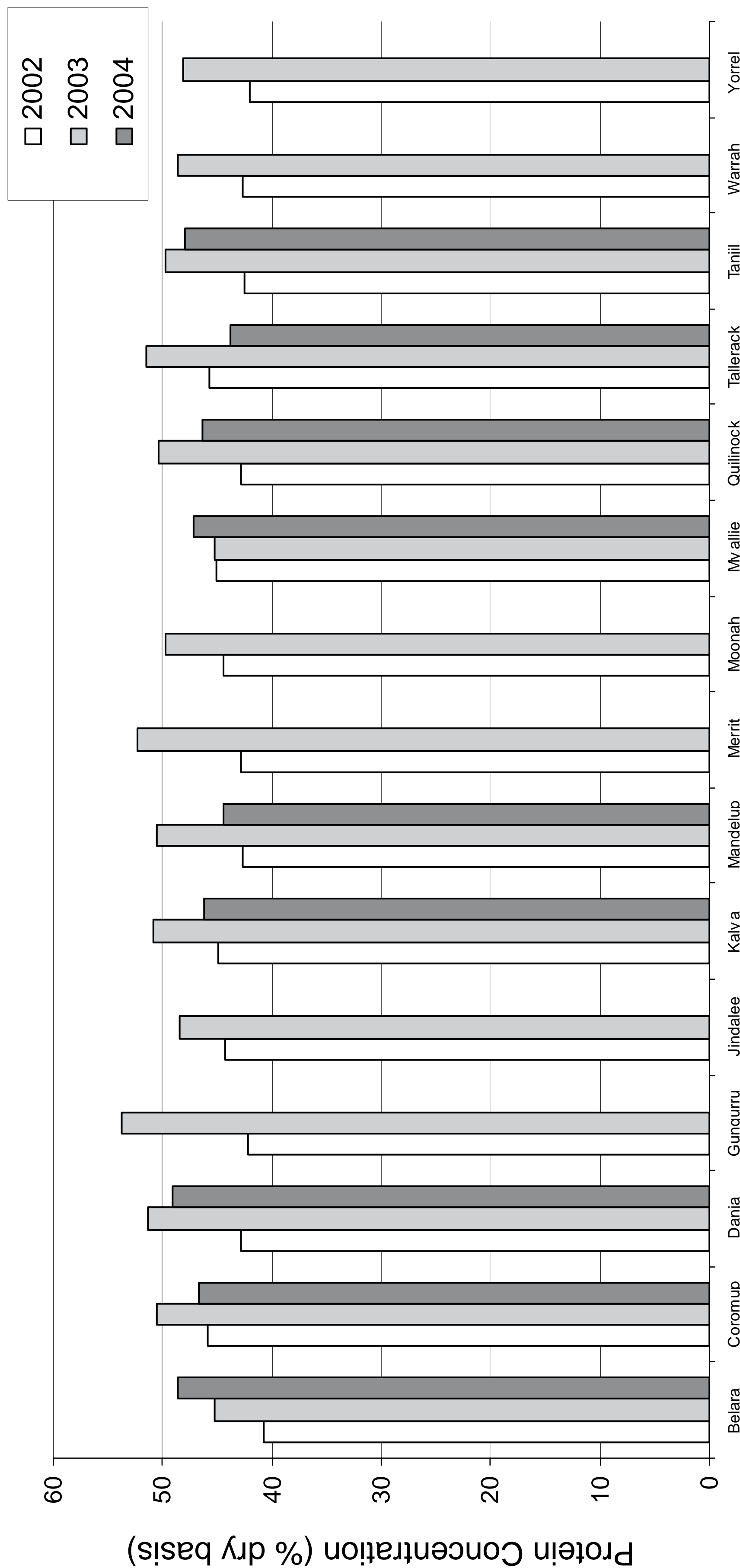

Figure 6.3 Variation in composition of each of the 15 commercial *L. angustifolius* cultivars across three years, all grown at the same site (Wongan Hills, Western Australia). Values are means.

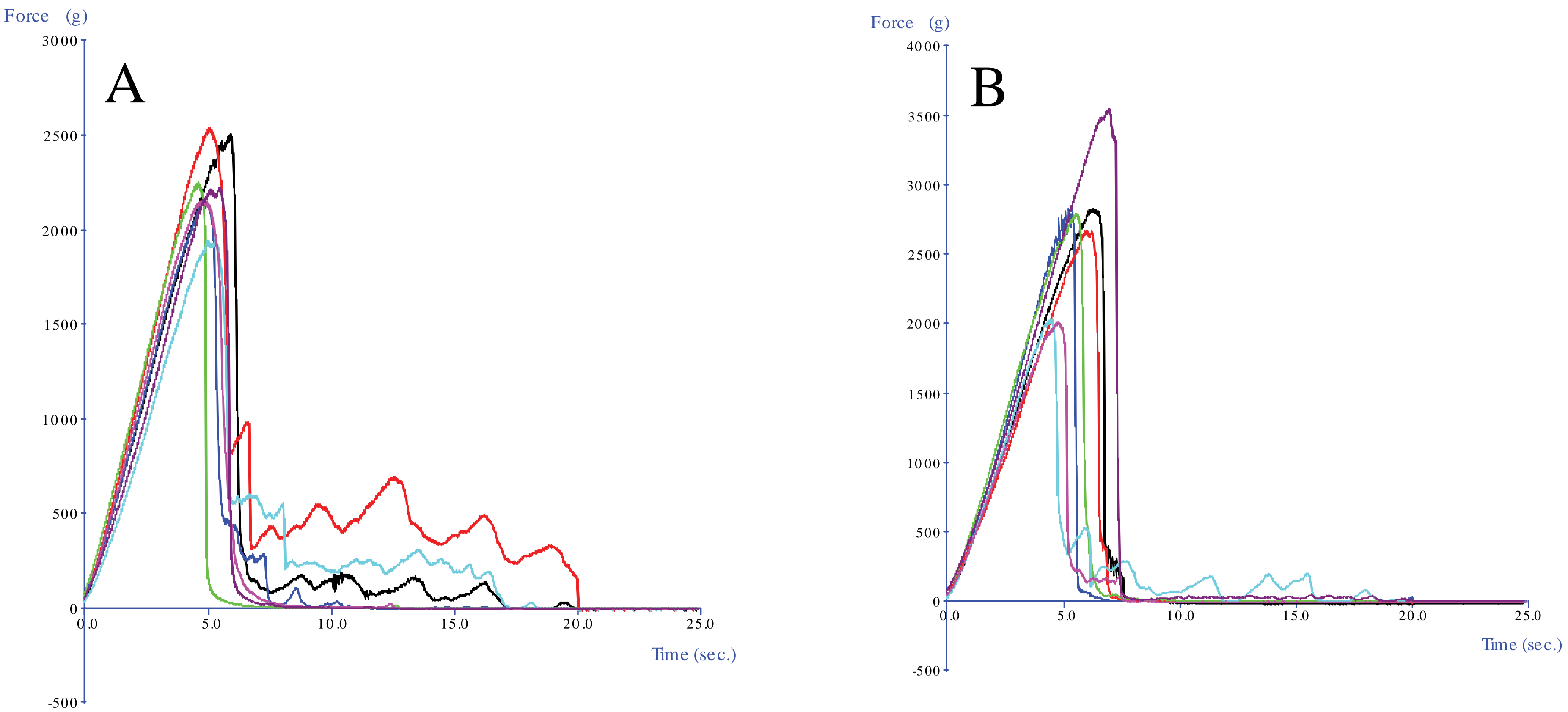

Figure 6.4 TA.XT2i texture analyser response to *L. angustifolius* cv. Mandelup (A) and cv Coromup (WALAN2173) (B) kernels. Notable is the different peak force required to cleave the kernels between the two cultivars.

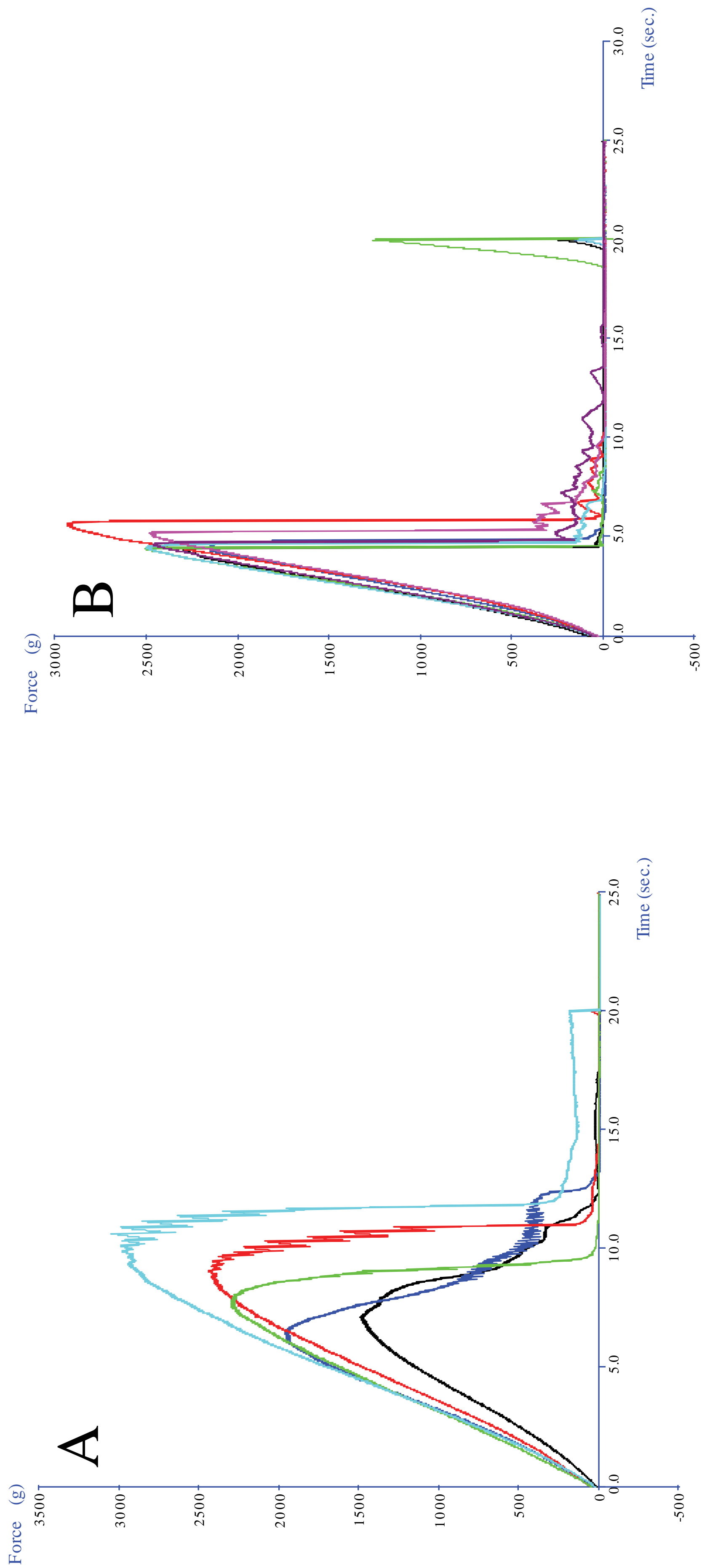

Figure 6.5 TA.XT2i texture analyser response to *L. angustifolius cv.* Belara (A) and *L. luteus cv* Wodjil (B) kernels. Notable is the different peak force required to cleave the kernels and also the difference in rate of force application (initial slope) between the two cultivars.

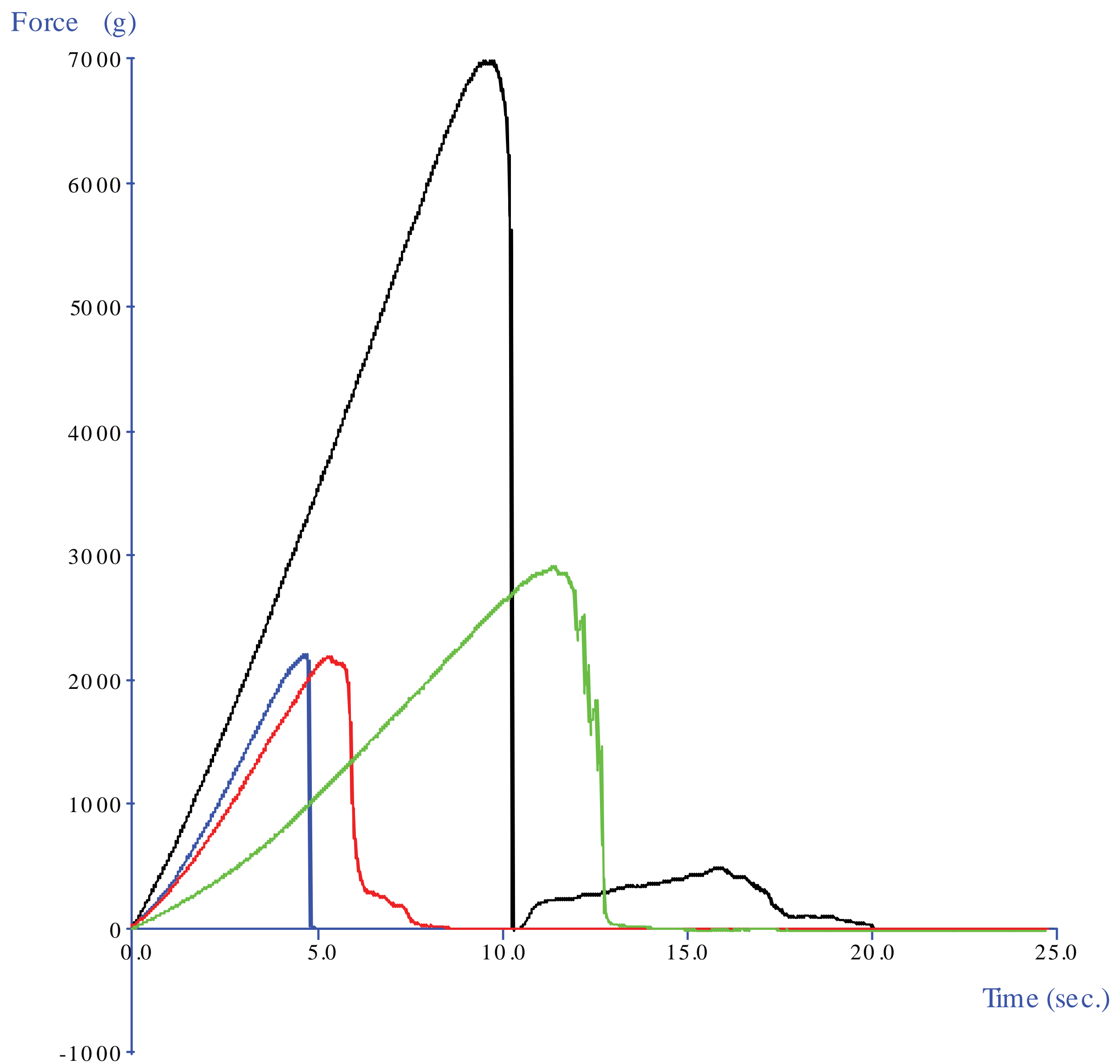

Figure 6.6 TA.XT2i texture analyser response to *L. angustifolius cv*. Belara (Green), *L. angustifolius cv*. Myallie (Red), *L. albus* cv. Kiev mutant (Black) and *L. luteus* cv Wodjil (Blue) kernels. Notable is the different peak force required to cleave the kernels and also the difference in rate of force application (initial slope) between the cultivars.

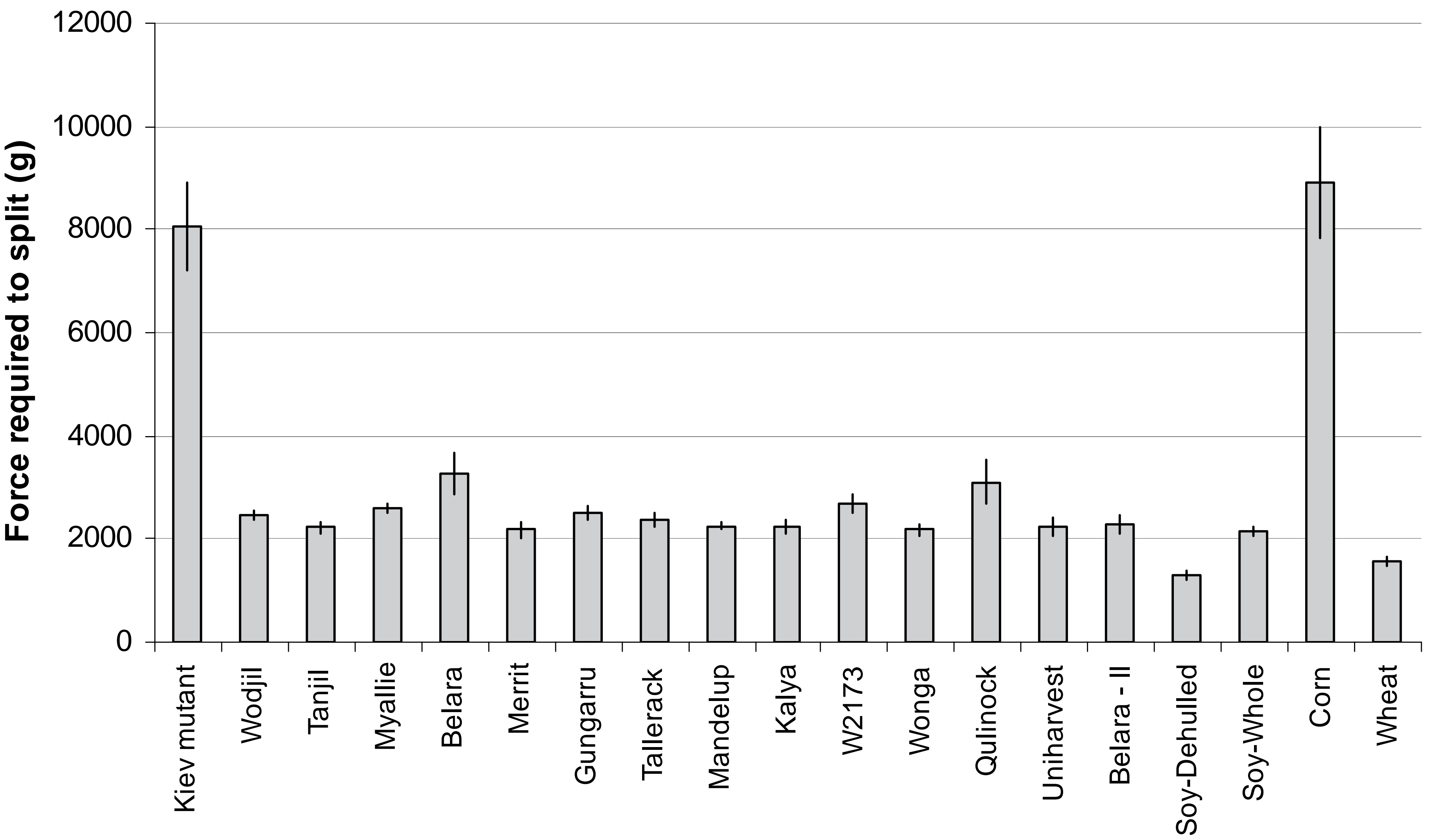

Figure 6.7 Force measured by the TA.XT2i texture analyser to cleave/split kernels from a range of lupin varieties and also soybeans, corn and wheat.

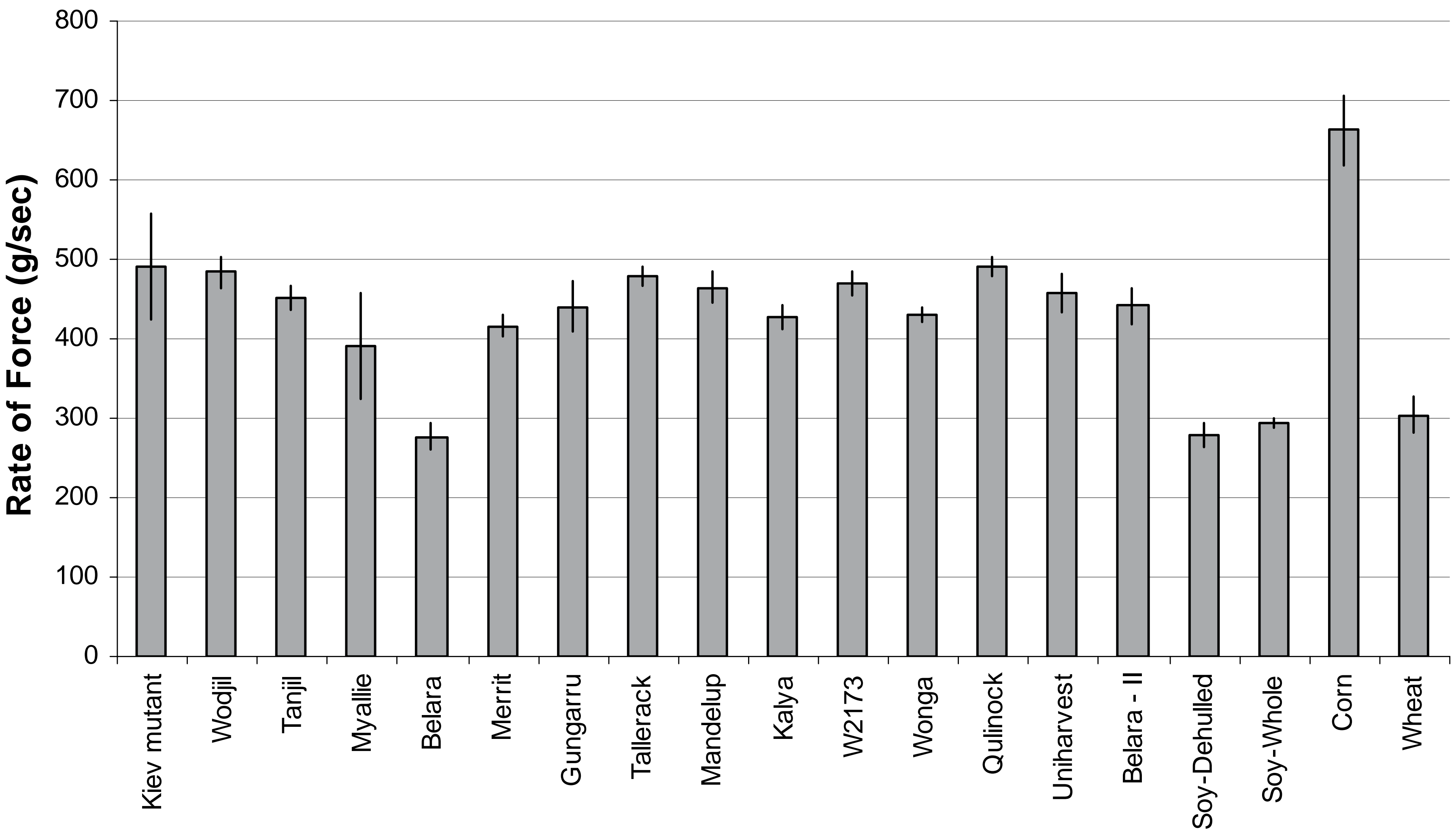

Figure 6.8 Rate of force application measured by the TA.XT2i texture analyser to cleave/split kernels from a range of lupin varieties and also soybeans, corn and wheat.

7.0 Assessing the variability of nutrient and energy digestibilities of lupin (*Lupinus angustifolius*) kernel meal when fed to rainbow trout (*Oncorhynchus mykiss*)

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Abstract

This study examined the variability in the digestibility of a range of lupin kernel meals when fed to rainbow trout. Over a series of seven separate experiments 75 different lupin kernel meals were assessed for their digestible dry matter, protein, amino acid and energy characteristics. A common reference basal diet and a reference lupin kernel meal diet were also included in each experiment. Minimal variance in the digestibility parameters of both reference diets was observed among the experiments ensuring that there was a high degree of robustness in the across-experiment evaluations. A slightly larger degree of variance was observed among the ingredient reference assessment, consistent with the amplification of errors that occurs with derived terms such as ingredient digestibility coefficients. However, even the ingredient digestibility variance was relatively low (< 10%) testifying to the high fidelity of the inter-experiment data. Using simple regression and multiple-regression techniques, principal diet and ingredient composition factors affecting diet and ingredient digestibilities and ingredient digestible values were explored with the dataset. Nitrogen digestibility of the lupin kernel meals was negatively influenced by ingredient lignin content, but positively affected ingredient fat content. There were no significant correlations between ingredient composition and sum of amino acids digestibility of the lupin kernel meals. The energy digestibility was positively affected by a range of kernel meal compositional features including protein, sum of amino acids and negatively affected by carbohydrate content. The digestible nutrient and energy content of the kernel meals reflected the combined effects of both ingredient digestibilities and ingredient composition. The digestible nitrogen content of the kernel meals was positively affected by protein, sum of amino acids and energy content, but was negatively affected by lignin and carbohydrate content. The digestible sum of amino acids was also positively affected by protein, sum of amino acids, but only negatively affected by carbohydrate content, not lignin content. The digestible energy content of the kernel meals was also positively affected by protein, sum of amino acids and its own energy density, but only negatively affected by carbohydrate content. Multiple regression modelling supported that together that ingredient protein and lignin content were the strongest predictors of digestible protein value, explaining close to 60% of the variability in this parameter. This study demonstrates that within one raw material type that not only does significant variability in the digestible value of the raw materials exist, but that it is possible to identify compositional features of that raw material that are intrinsically influencing its own digestible value. This feature has the potential to be applied to rapid analysis techniques, such as near infrared spectroscopy to allow the development of calibrations to predict digestible

values of both diets and raw materials and also provides some basis by which higher values can be ascribed to better quality lupin kernel meals.

7.1 Introduction

Considerable research has been undertaken to identify and evaluate alternatives to fishmeal for use in diets for many aquaculture species (Moyano et al., 1992; Gomes et al., 1995, Suigura et al., 1998; Carter and Hauler, 2000; Storebakken et al., 2000). Of those studies reported, lupins are one raw material that has been shown to provide a sound prospect for use in fish diets.

Like all raw materials, the composition of *L. angustifolius* can vary considerably depending on growing season attributes, cultivar and soil conditions (Longnecker et al., 1998). This variability is normally managed by large scale blending of grain received from growers at centralised receival points (Perry et al., 1998). This variability in composition has also been noted to extend to the digestible value of lupin kernel meals (Glencross et al., 2003a; Glencross and Hawkins, 2004). The nutritional value of lupin grain, and indeed, that of most plant proteins is usually a direct reflection of their digestible protein and/or energy content (Burel et al., 1998; Glencross et al., 2004; Glencross et al., 2005). Accordingly any variability in the digestible value of the meals should translate to variability in their economic value. Recently, the increasing adoption of lupin kernel meal use by the aquaculture feed sector has encouraged the introduction of segregation and premiums for higher protein content in *L. angustifolius* grain.

In lupins, an increase in protein content is usually offset by a concomitant decrease in the levels of non-starch polysaccharides (NSP) (van Barneveld, 1999c; Petterson, 2000). High levels of NSP and other fibre types have been implicated in reduced nutritional value of plant protein meals (Arnessen et al., 1989; Refstie et al., 1998; Glencross et al., 2003b). Furthermore, because lupins are largely devoid of starch it is hypothesised that only the protein and lipid components of the raw material are contributing to its nutritional value (Glencross et al., 2007b). However, the specific compositional features of lupin kernel meals that actually are actively affecting their digestible nutrient and energy values remain to be conclusively defined (van Barneveld, 1999a). Given that modern aquaculture diets are formulated on a digestible nutrient and energy basis, then better assessment of the value of the raw material on this basis will provide significant cost savings in diet formulation.

The ability to chemically identify factors within raw materials that affect nutrient and energy digestible values lends itself to development of further raw material assessment methods (King and Taverner, 1975; van Barneveld, 1999a; 1999b). Notably, the use of near-infrared spectroscopy (NIRS) to predict digestible values based on differences in compositional variability is one possibility (van Barneveld et al., 1998; Bertrand, 2001). Such an ability to more accurately measure the digestible nutrient and energy value of a raw material will allow formulators to tighten diet specifications and ultimately reduce the cost of their formulations. Presently this uncertainty in raw material quality is managed by over-specifying nutrients and energy in the formulation.

This study reports on the evaluation of the variability in the digestibility of kernel meals of narrow-leaf lupins, *Lupinus angustifolius* when fed to rainbow trout (*Oncorhynchys mykiss*). The variability is further examined as a function of the influence that each kernel meal has on the composition of the diet and also how that composition affects its own nutritional value.

7.2 Materials and Methods

7.2.1 Ingredient and diet development

Over a three-year period, separate batches of seed of *Lupinus angustifolius* were collected from the Department of Agriculture's (WA) germ plasm and breeding lines. This seed in many cases constituted the same genotype over several seasons, often from the same site. Samples of the seed were then split using a small disc-mill and aspirated to separate hulls from kernels. A final manual clean of the kernels to remove any remaining hull material was also undertaken on each sample to ensure 100% purity of the kernel preparation. Each kernel sample was then milled using a Restsch rotor mill with a 750 µm screen to create a kernel flour. In addition to the lupin kernel flours, each of the test ingredients used in this study was thoroughly ground such that they passed through a 750 µm hammer mill screen.

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 7.2). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 7.2). Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. An additional reference lupin kernel meal was included in every digestibility study to allow for cross-comparison across all studies. The basal diet and an example test diet formulations and their composition are presented in Table 7.2.

7.2.2 Fish handling and faecal collection

These digestibility studies constituted seven separate experiments. Each experiment had two common diets, which included the reference diet and a reference lupin kernel meal (Myallie). For each experiment hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 l) between three and ten days prior to being introduced to the experimental diets. Freshwater (salinity < 1 PSU) of 16.0 ± 0.1°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. For each experiment the tanks were stocked with 15-20 trout of 254± 62.5 g (mean ± S.D.; n = 7 experiments). Treatments were randomly assigned amongst 48 tanks within each experiment, with each treatment having three replicates.

Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600hrs each day. The trout were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not

contaminated by urine or mucous. The hands of the person stripping the fish were rinsed with freshwater between each fish. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and stored in a freezer at -20°C and the fish returned to its treatment tank to revive. Stripped faeces were collected during 0800 to 1000hrs over a four-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples from different days were pooled within tank, and kept frozen at -20°C before being freeze-dried in preparation for analysis.

7.2.3 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by McQuaker et al. (1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Amino acid composition of samples was determined by an acid hydrolysis prior to separation via HPLC. The acid hydrolysis destroyed tryptophan making it unable to be determined using this method. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the method of Folch et al. (1953). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Dietary fibres were determined by digesting the defatted sample with multiple washes of acetone and ethanol. The resulting residue was corrected for undigested protein and ash according to the method of the Champ, et al.(1998). Neutral-detergent fibre (NDF) samples were boiled with buffered NDF solution. The residue is collected on a coarse sintered glass crucible (Van Soest and Robertson, 1981). The acid-detergent fibre (ADF) was determined following a sample being reacted in 0.5M acid detergent solution and the residue is collected on a coarse sintered glass crucible after, the method of Van Soest and Goering (1970). Lignin is determined by reacting the ADF residue with cold 72% sulphuric acid. The sample is ashed and the residue measured gravimetrically (Van Soest and Robertson, 1981). Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

where Y_{diet} and Y_{faeces} represent the chromium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 7.4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%.

7.2.4 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Figures were constructed using Microsoft Excel. Single parameter correlation analysis and multiple regression analysis was undertaken using Statitistica v6. Limits for all critical ranges were set at $P < 0.05$. Because of nominal variance in the data, no standardisation of the inter–experiment data was required.

7.3 Results

7.3.1 Data variance

Over a series of seven independent experiments both the basal reference and ingredient reference diets had minimal variability in their digestibility parameters among experiments (Table 7.2). Dry matter diet digestibilities were different for both diets, but had a similar coefficient of variance of 2.2%. Coefficients of variance (CV) for diet protein digestibility were low at 0.9% and 1.3%, but the means were similar. Diet energy digestibilities were different for both diets, but had a similar CV of 1.4 and 1.7%. Diet digestibilities of the sum of amino acids were similar for both diets, but had a similar CV of 0.9%, the lowest of the parameters evaluated.

Variability of the ingredient apparent digestibility coefficients for the reference ingredient were greater than that observed of the diet digestibilities (Table 7.2). Energy digestibility was the most consistent of the ingredient parameters evaluated, with a CV of 4.2%. Ingredient digestibilities for the Sum of Amino acids had the highest variability with a CV of 20.6%.

Variability of the composition of the lupin kernel meals used in this study is presented in chapter 6. As a summary of that data; the mean ± S.D., protein (N x 6.25) concentration in lupin kernels, across all 75 samples was 45.4 ± 3.4% on a dry basis (range 36.55 to 56.7%). Total lipid was 7.8 ± 0.9% (range 5.2% to 9.7%) and ash 3.0 ± 0.4%. Carbohydrates, measured by difference between dry matter minus protein, lipid and ash, were 43.8 ± 3.3% on a dry basis (range 32.7% to 53.9%). Mean gross energy was 20.8 ± 0.3 MJ/kg DM (range 20.1 to 21.5 MJ/kg DM). Dietary crude fibre was 30.9 ± 4.6% on a dry basis (range 17.5% to 43.8%), acid-detergent fibre was 10.2 ± 5.3% on a dry basis (range 5.2% to 26.2%), neutral-detergent fibre was 6.6 ± 4.5%

on a dry basis (range 3.0% to 20.0%) and lignin was 0.7 ± 0.5% on a dry basis (range 0.2% to 2.2%) (Table 7.4).

7.3.2 Diet digestibility coefficients

Substantial variability in most diet digestibility parameters was measured across all experimental diets (Table 7.3). Phosphorus digestibility was the most variant of the diet digestibility parameters with a coefficient of variation of 12.2%. Most other diet digestibility parameters had coefficients of variation less than 5%. The key digestibility parameter of diet nitrogen digestibility had a coefficient of variation of 1.0%, with a range in apparent nitrogen digestibilities of 0.881 to 0.923 (Table 7.3). The key digestibility parameter of diet energy digestibility had a coefficient of variation of 1.9%, with a range in apparent energy digestibilities of 0.785 to 0.861 (Table 7.3). The key digestibility parameter of diet sum of amino acids digestibility had a coefficient of variation of 0.9%, with a range in apparent sum of amino acids digestibilities of 0.912 to 0.945 (Table 7.3). This variability in diet digestibility parameters is an effect of the variability in ingredient values, not the assessment methods as demonstrated in Table 7.2.

The only diet compositional parameters that correlated with diet nitrogen digestibility were diet fat content and the sum of amino acids content (Table 7.4). Similarly, the sum of amino acids digestibility also only significantly correlated with diet fat content, but not diet sum of amino acids content.

Dietary energy digestibility was significantly affected by the diet carbohydrate density (R=-0.2691, P= 0.014) and the diet protein+fat density (R=0.4105, P=0.000) (Table 7.3, Figure 7.2). There was no significant effect of diet protein density alone (R=0.1610, P=0.148) on diet energy digestibility, nor was there any effect of diet energy density (R=-0.0957, P=0.393) on the energy digestibility of the diets.

To confirm logical relationships expected to occur within the diet digestibilities, the relationship between digestibility coefficients of protein (nitrogen) and protein (sum of amino acids) were examined (Figure 7.3). This was found to be a highly significant relationship (R=0.8678, P=0.000). The relationship between the diet digestibility coefficients of protein (nitrogen) and energy was examined (Figure 7.3). This was also found to be a highly significant relationship (R=0.6553, P=0.000).

7.3.3 Ingredient digestibility coefficients

A greater level of variability in most ingredient digestibility parameters compared to those in the complete diets, was measured across all experimental ingredients (Table 7.5). Fat digestibility was the most variant of the ingredient digestibility parameters with a coefficient of variation of 80.7%. The ingredient nitrogen digestibility had a coefficient of variation of 10.3%, with a range in apparent nitrogen digestibilities of 0.655 to 1.146 (Table 7.5). The ingredient energy digestibility had a coefficient of variation of 8.0%, with a range in apparent energy digestibilities of 0.482 to 0.694 (Table 7.5). The ingredient sum of amino acids digestibility had a coefficient of variation of 14.8%, with a range in apparent sum of amino acids digestibilities of 0.526 to 1.265 (Table 7.5). This variability in ingredient digestibility parameters is an effect of the variability in ingredient values, not the assessment methods as demonstrated in Table 7.2.

Lupin kernel meal protein (nitrogen) digestibility was not significantly affected by the ingredient protein density (R=-0.2946, P= 0.086) or the ingredient carbohydrate density (R=-0.2055, P=0.236) (Table 7.4, Figure 7.4). Neither was there any significant effect of ingredient crude fibre,

acid-detergent fibre or neutral-detergent fibre density on ingredient protein digestibilities (Table 7.4, Figures 7.6, 7.7). Ingredient lignin content however, had a significant effect on ingredient protein digestibility (R=-0.7036, P=0.000), of the lupin kernel meals (Table 7.4, Figure 7.7).

Lupin kernel meal energy digestibility was significantly affected by a wider variety of ingredient composition parameters (Table 7.4). The ingredient protein (nitrogen) density (R=0.4659, P= 0.005) significantly positively influenced the energy digestibility coefficient of the ingredient. The ingredient protein density measured as sum of amino acids even more significantly positively influenced the energy digestibility coefficient of the ingredient (R=0.5694, P= 0.000) than that estimated by nitrogen. The ingredient protein+fat density (R=0.4738, P=0.004) had a stronger significantly positive influence on the energy digestibility coefficient of the ingredient. Reciprocating this, the ingredient carbohydrate density (R=-0.4904, P=0.003) had a significant negative effect on ingredient digestibility. Ingredient energy density had no effect (R=0.2343, P=0.176) on the ingredient energy digestibility coefficient. There was no significant effect of ingredient crude fibre, acid-detergent fibre, neutral-detergent fibre density or lignin content on the ingredient energy digestibilities (Table 7.4, Figures 7.10, 7.11).

7.3.4 Ingredient digestible values

Substantial variability in ingredient digestible nutrient parameters was measured across all experimental ingredients (Table 7.5). This variability was compounded by the variability in ingredient composition and ingredient digestibility. The digestible nutrient parameters had coefficients of variation ranging from 8.2% for digestible energy to 55.1% for digestible lipid. The key digestibility parameter of ingredient digestible nitrogen had a coefficient of variation of 11.3%, with a range in digestible nitrogen levels of 30.4 to 54.7 (Table 7.5). The ingredient digestible energy levels had a coefficient of variation of 8.2%, with a range in ingredient digestible energy of 9.9 MJ/kg to 14.5 MJ/kg (Table 7.5). The ingredient digestible sum of amino acids digestibility had a coefficient of variation of 16.1%, with a range in ingredient digestible sum of amino acids of 23.4 to 50.6 (Table 7.5). This variability in ingredient digestible nutrient parameters is an effect of the variability in ingredient values, not the assessment methods as demonstrated in Table 7.2.

Lupin kernel meal digestible protein (nitrogen digestibility x meal protein content) was significantly affected by the ingredient protein density (R=0.4109, P= 0.014) and by reciprocation the ingredient carbohydrate density (R=-0.4921, P=0.003) (Table 7.5, Figure 7.12). The sum of amino acids in the ingredient also correlated strongly with the digestible protein value (R=0.4372, p=0.009). The relationship between protein content and energy also meant that energy density was a significant correlate to digestible protein value (R=0.4836, p=-0.003). There was no significant effect of ingredient crude fibre, acid-detergent fibre or neutral-detergent fibre on ingredient protein digestibilities (Table 7.5, Figures 7.13). However, lignin content of the lupin kernel meals had a significant (R=-0.04981, p=0.002) effect on the level of digestible protein in the kernel meals.

Lupin kernel meal digestible sum of amino acids was significantly affected by the ingredient protein density (R=0.7197, P= 0.000) and by reciprocation the ingredient carbohydrate density (R=-0.4921, P=0.003) (Table 7.5, Figure 7.12). The sum of amino acids in the ingredient also correlated strongly with the digestible sum of amino acids value (R=0.5801, p=0.000). The energy density was not a significant correlate to digestible sum of amino acids value (R=0.3066, p=-0.073). There was no significant effect of ingredient crude fibre, acid-detergent fibre, neutral-detergent fibre or lignin density on ingredient protein digestibilities (Table 7.5).

Lupin kernel meal digestible energy value was also significantly affected by a wide variety of ingredient composition parameters (Table 7.5). The ingredient protein (nitrogen) density (R=0.4978, P= 0.002) significantly positively influenced the digestible energy content of the ingredient. The ingredient protein density measured as sum of amino acids even more significantly positively influenced the energy density of the ingredient (R=0.6192, P= 0.000) than that estimated by nitrogen. The ingredient protein+fat density (R=0.5368, P=0.001) had a stronger significantly positive influence on the digestible energy density of the ingredient than the protein content alone (Table 7.5, Figure 7.14). Reciprocating this, the ingredient carbohydrate density (R=-0.5421, P=0.001) had a significant negative effect on digestible energy levels. The ingredient energy density had a significant effect (R=0.4164, P=0.013) on the digestible energy level of the ingredient. There was no significant effect of ingredient crude fibre, acid-detergent fibre, neutral-detergent fibre density or lignin content on the ingredient energy digestibilities (Table 7.5, Figure 7.15).

7.4 Discussion

Variability exists in all ingredients. This variability can be managed through a variety of means, either by the ingredient supplier, or by the feed manufacturer. Examples of this include the large-scale blending by commodity handlers of grains of different protein levels to produce a more homogenous product, or the analysis of batch variation by feed manufacturers to allow precise customisation of each diet according to each batch of ingredients supplied (Jiang, 2001; van Barneveld, 2001). In addition to these ingredient management strategies an improved understanding of the level of variability in the chemical composition of the ingredient and how that variability contributes to changes in nutritional value is a key step to maximising the potential value of the ingredient. In this study a series of 75 *Lupinus angustifolius* kernel meal samples were collected over a three-year period and examined in a series of digestibility assays with rainbow trout. The composition of each of the kernel meals varied substantially and this variability was used to assess the compositional features of the grain that affected their nutritional value using a regression modelling approach adapted from nutritional studies with terrestrial species (Harris et al., 1972; Bhatty et al., 1974; King and Taverner, 1975; Bell et al., 1983; Fairbairn et al., 1999).

7.4.1 Influence of diet composition on diet digestibility

Although the strategy used in diet formulation in this study was to replace 300 g/kg of the reference diet with each test ingredient, the variability in test ingredient composition resulted in a nominal level of variability in key diet composition parameters. Parameters like diet protein varied from 45.0% to 55.1%, with a coefficient of variation of 3.9%, while diet energy content varied from 22.1 to 23.0 MJ/kg DM with a coefficient of variation of 0.9% (Table 7.3).

There was significant variability between diets in the digestibility of most diet parameters, but limited variability of those parameters within diets (Table 7.2 and Table 7.3). This variability was primarily attributable to the variance in digestibility value of the test lupin kernel meals.

There were some effects of diet composition on diet digestibility parameters. It was observed that diet nitrogen digestibility was correlated with the diet fat content and the sum of amino acids content (Table 7.4). Similarly, the sum of amino acids digestibility was also significantly correlated with diet fat content, but not the diet sum of amino acids content. The limited variability in diet nitrogen and sum of amino acids parameters probably contributed to no observable significant effects. A broader range of diet protein levels may have had more influence on this

parameter (Glencross et al., 2007), but clearly the object of the present study was to limit diet effects to enable a focus on ingredient effects.

The dietary energy digestibility was significantly affected by the diet carbohydrate density, diet fat density and protein+fat density but not protein density alone (Table 7.3). That there was no significant effect of diet energy density on the energy digestibility of the diets, despite that there were significant effects from fat, protein+fat and carbohydrate is interesting, but suggests that the variability in energy density was insufficient to enable useful correlations to be drawn.

This study also reports one of the few pieces of work to examine digestibility of the same diet across many experiments (n=7) across several years (n=3). It was observed that over a series of seven independent experiments that both the basal reference and ingredient reference diets had minimal variability in their digestibility parameters among experiments which we believe demonstrates that there was a high degree of precision in the digestibility assessments undertaken in this work (Table 7.2). Notably, the coefficients of variance for each parameter were well below 5%. As is to be expected, the variability of the ingredient apparent digestibility coefficients for the reference ingredient were greater than that observed of the diet digestibilities (Table 7.2). Only the ingredient digestibilities for the sum of amino acids, with a CV of 20.6%, could potentially be regarded as highly variable. No other references to other such similar work could be found to be of comparison to this study to gauge the relative degree of fidelity of this work.

7.4.2 Influence of ingredient composition on ingredient digestibility and digestible values

Any compound feed for an animal is generally only as valuable as the sum of the value of its ingredients. The key value in an ingredient such as lupin kernel meal is its protein and/or energy content. Although the assessment of protein can be made using different methods (e.g. nitrogen x 6.25 or sum of amino acids) and this in its own right may affect the assessment process (Glencross et al., 2007a). Lupin kernel meals, like all ingredients, also possess an inherent amount of variability in their composition. In the current study, protein levels of the *L. angustifolius* kernel meals ranged from 36.5%DM to 56.7%DM. In each case, the changes in protein content of the kernel meals were concomitant with changes in the carbohydrate (CHO) content of the kernel meals, as limited variability in the fat or ash content of the meals was observed. This is consistent with what has been reported in other studies (Petterson et al., 1997; Glencross et al., 2003a). Although the variability in dietary crude fibre (range 17.5% to 43.8%), acid-detergent fibre (range 5.2% to 26.2%), neutral-detergent fibre (range 3.0% to 20.0%) and lignin (range 0.2% to 2.2%) was substantially greater in comparison to the other key proximate parameters. This variability in the compositional parameters enhanced the ability of the study to identify some likely compositional factors that were related to variability in digestibility coefficients and digestible values.

As was expected, there was a greater level of variability in the ingredient digestibility parameters compared to those in the complete diets (Table 7.5). Although fat digestibility was the most variant of the ingredient digestibility parameters with a coefficient of variation of 80.7%, this was probably an artefact of its low levels in the test ingredients relative to the diets and also the low levels of residual fat in the faeces resulting in a more variable assessment as much as anything. The small faecal samples used for fat analysis also probably increased the risk of error. Generally, variability in most ingredient digestibilities, like that of nitrogen digestibility had a coefficients of variation closer to 10%, but still with a substantial range in apparent nitrogen digestibilities that made for useful correlation and multiple regression analyses (Table

7.5 and 7.7). The variability in both these ingredient digestibilities and the composition of the ingredients themselves compounded to increase the overall variability observed in the digestible nutrient values of the lupin kernel meals.

In contrast to earlier findings (Glencross et al., 2003a) the lupin kernel meal nitrogen digestibility was not significantly affected by the ingredient nitrogen density or the ingredient carbohydrate density (Table 7.4, Figure 7.4). However, the finding that ingredient lignin content did have a highly significant effect on ingredient nitrogen digestibility of the lupin kernel meals is an important finding. This observation is consistent with other studies on cattle, pigs and poultry that have also reported that the presence of lignin affects digestibility parameters (Crampton and Maynard, 1938; King and Taverner, 1975), but this is the first such observation with fish. In contrast to the effects seen in pigs fed barley, there was limited response of the digestibility parameters in fish to levels of ADF in the lupin kernel meals (Fairbairn et al., 1999).

The energy digestibility of the lupin kernel meals was significantly affected by a wider variety of ingredient composition parameters than that observed for nitrogen digestibility (Table 7.4). The observation that ingredient nitrogen density significantly positively influenced the energy digestibility coefficient of the ingredient was consistent with earlier studies (Glencross et al., 2003a). The ingredient protein density measured as sum of amino acids even more significantly positively influenced the energy digestibility coefficient of the ingredient than that estimated by nitrogen. This observation draws to attention the possible irregularities associated with relying on either method of protein measurement. The strong effect of the ingredient protein+fat density on the energy digestibility coefficient of the ingredient was also reciprocated by the ingredient carbohydrate density effect, which had a significant negative effect on ingredient digestibility. Notably, the addition of the ash variability to the carbohydrate assessment further increased the robustness of the correlation. In this regard it is probably that the protein+fat may be partly a reciprocated effect of the carbohydrates, fortified by an effect of protein content.

One risk, though, of drawing conclusions about the role of lupin carbohydrates (CHO = dry matter – protein – ash – lipid) in the digestibility assessment is that their determination is based on that of the other key nutrients of protein, lipid and ash. Therefore any variability relating to a carbohydrate effect cannot be distinguished from a combined or partial effect of the other nutrients. Therefore, further assessment has been made of certain fibre classes within a sub-set of the lupin samples to explore the carbohydrate factor more fully. The fibre content of lupins consists largely of non- starch polysaccharides, which is a generic term for other components such as cellulose, lignin, pectins, dextrins, inulin, beta-glucans and oligosaccharides (Englyst, 1989; Petterson et al., 1997).

The use of both two-way regression and step-wise regression analysis allowed the exploration of multiple factors in influencing the ingredient digestible values (Table 7.9 and 7.10). It is apparent that multiple parameters are simultaneously affecting the digestible values of lupin kernel meals. Most notable was the dual effect of both ingredient protein and lignin effect in affecting the digestible value of the protein (irrespective of whether analysed as nitrogen or sum of amino acids).

In the present study it was found that crude fibre had little effect on any digestibility or digestible value parameter of the lupin kernel meals. Notably, crude fibre analysis is now regarded as a largely redundant assessment that provides little meaning as the actual carbohydrate/fibre chemistry of the plant (Petterson et al., 1999). An assessment of acid-detergent fibre (ADF), or neutral-detergent fibre (NDF) and lignin is now regarded as more meaningful, with the measurement of each parameter allowing the determination of the cellulose, hemicellulose and lignin contents of the sample polysaccharides (Hindrichsen et al., 2006).

The observation that the lignin class of polysaccharides was a key factor in affecting digestibility responses in fish is significant new finding, which identifies a specific fibre class as having anti-nutritional benefits. The level of lignin in the lupin kernel meals was observed to directly correlate with a decline in nitrogen digestibility and also the overall digestible nitrogen/protein value of the kernel meals. This relationship was one of the strongest observed in the study (Table 7.7 and 7.8). Further examination of the influence of lignin showed that based on multiple and/or step-wise regression techniques, that lupin kernel meal protein and lignin content together accounted for close to 60% of the variability in digestible protein (as either Nx6.25 or sum of amino acids) value of these grains.

These observations noted in the current study are also supported by an increasing volume of literature that suggests that non-starch polysaccharides (NSPs) in general reduce the nutritional value of plant protein meals fed to fish (Arnessen et al., 1989; Refstie et al., 1998; 1999; Glencross et al., 2003b; Glencross et al., 2005). Notably, studies with Atlantic salmon have identified that the NSP from soya beans, which are similar to those in lupins, had an influence on the nutritional value of soybean protein (Refstie et al., 1999; Petterson, 2000). Moreover, an earlier study by Arnessen et al. (1989) examined ethanol extracted soya bean meal and showed that the ethanol extracted soya bean meal had improved nutritional value as a consequence of the ethanol extraction. Notably, the ethanol extraction process most likely removed the soya bean oligosaccharides, but probably also removed other anti-nutritionals like saponins (Coon et al., 1990). Later work by Glencross et al. (2003b) also showed that both ethanol extraction and enzymatic hydrolysis of α-galactosides significantly improved the digestion of energy and protein from both *L. angustifolius* by fish. This supported the hypothesis that oligosaccharides could interfere with digestion of other nutrients when fed to fish, and suggests that the oligosaccharide content of lupins may also be influencing the nutritional value of its own protein.

7.4.3 Assignment of value to protein levels

The key value in an ingredient such as lupin kernel meal is its protein content. Accordingly, the higher the protein content of the kernel meal, then the greater the value of that resource. This proposition is founded on two aspects. One that the higher the protein content of the meal, then the more flexibility the ingredient provides in formulating diets for fish. Second, the current study shows that as the protein content of the lupin kernel meal increases, then so too does the digestibility of its protein and the amount of protein available to animal. From the present work this relationship can be described by the equation: $y = 0.5858x + 15.707$, $R^2 = 0.1795$. However, this equation is rather nonsensical as it suggests at protein levels below 37% that the digestible protein is also greater than the crude protein content of the meal and that protein digestibility in low-protein lupin kernel meals is greater than that in high-protein lupin kernel meals and. This is the opposite of earlier assessments done on a similar basis (protein digestibility with varying kernel meal protein content), but with a significantly smaller sample set (n=5 vs n=75) (Glencross et al., 2003b). Based on other more recently determined relationships between lupin meal protein content and protein digestibility (Glencross et al., 2007c), a non-linear relationship is proposed whereby: $y = -0.0449x^2 + 4.7609x - 80.673$, $R^2 = 0.2093$. Notably this non-linear relationship is also stronger than the initial linear one proposed and accordingly suggests that other relationships too might be better examined with non-linear models. The combination of a non-linear protein relationship with a linear lignin relationship results in the function: Digestible protein = $-0.0449x^2 + 4.7609x - 80.673)\bullet(-0.1956y + 1.052)$, where x is the kernel meal crude protein content and y is the kernel meal lignin content (Figure 7.17).

Based on the identified relationships between the digestible value of lupin kernel meals and their composition, it may be possible to develop calibrations for near-infrared spectroscopy (NIRS) to be able to rapidly measure digestible values of these ingredients (van Barneveld et al., 1998; Bertrand, 2001). Calibrations that used dual assessment of compositional parameters would be likely to be more successful than single parameter calibrations. Calibrations based on crude protein and lignin content of the kernel meals is suggested. The development of a calibration for digestible protein would have substantial benefit for both the grains processing and the aquaculture feed sectors, where by each sector could more accurately assess the actual value of their raw materials prior to sale and use respectively.

7.4.4 Conclusion

From this study it was further identified that there was a strong correlation between protein content (as assessed based on either Nx6.25 or sum of amino acids) of a lupin kernel meal and its digestible value but that additional features of the grain composition, such as lignin, also affect this digestible value. Further exploration of the complexity of polysaccharides in grains and how these relate to nutritional value of those grains may be warranted.

That the level of both nitrogen and energy digestibility from the lupin kernel meals improves with increasing protein content provides good support for the development of lupin kernel meals with higher protein levels. Several prospects exist for improving the protein content of lupin meals including selective breeding of *L. angustifolius* varieties for protein content, improved efficiencies in the processing of the lupin seed to produce the kernel meal and the development of protein concentrates through air-classification and solvent extraction techniques.

The relationship between lupin kernel meal protein content and its digestible value also provides a good support for the development of a system of grain segregation by protein content and ingredient pricing according to that protein content. This would not only increase returns to grain producers but also more accurately reflect the actual value of the grain to its users. Additionally, the finding that a specific fibre class – lignin, affected the nutritional value of the lupin kernel meals more so that others provides an additional direction towards ways in which to higher quality lupin products can be targeted.

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Tables and Figures

Table 7.1 Diet formulations (all values are g/kg) and composition (n=7; all values are g/kg DM).

	Basal diet	Reference diet
Formulation		
Fishmeal [a]	700.0	490.0
Fish oil [a]	150.0	105.0
L. angustifolius kernel meal cv Myallie [b]	–	300.0
Wheat flour [a]	144.0	100.8
Vitamin and mineral premix a*	5.0	3.5
Yttrium oxide [c]	1.0	0.7
Composition		
Dry matter (g/kg)	953	945
Crude protein	510	498
Crude fat	228	178
Ash	124	98
Carbohydrate**	138	226
Gross Energy	23.3	22.5

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia.

[b] Supplied by Coorow Seed Cleaners Pty Ltd, Coorow, Western Australia, Australia.

[c] Supplied by Stanford Materials, Aliso Viejo, California, United States.

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

**Carbohydrate content determined based on dry matter minus protein, ash and fat.

Table 7.2 Mean values and data variance associated with apparent digestibility coefficients of the basal reference diet and the reference ingredient digestibility assessments across all experiments (n=7).

	Dry matter	Protein	Energy	Sum Amino Acids
Diet digestibility – Basal reference diet				
Mean	0.822	0.905	0.899	0.935
SD	0.019	0.012	0.013	0.008
SEM	0.007	0.004	0.005	0.003
CV%	2.3%	1.3%	1.4%	0.9%
Diet digestibility - *L. angustifolius* cv Myallie reference ingredient				
Mean	0.726	0.904	0.804	0.929
SD	0.016	0.008	0.013	0.008
SEM	0.006	0.003	0.005	0.003
CV%	2.2%	0.9%	1.7%	0.9%
Ingredient digestibility - *L. angustifolius* cv Myallie reference ingredient				
Mean	0.503	0.982	0.557	0.914
SD	0.039	0.072	0.023	0.188
SEM	0.015	0.027	0.009	0.071
CV%	7.7%	7.4%	4.2%	20.6%

CV%: Coefficient of variation = SD / Mean x100

Table 7.3 Variability in diet composition and digestibility parameters from all test diets. All values are g/kg DM unless otherwise detailed.

	MEAN	S.D.	CV%	MIN	MAX
Diet composition					
Dry matter (g.kg)	95.2	0.8	0.9%	92.4	96.8
Protein (N x 6.25)	49.6	2.0	3.9%	45.0	55.1
Fat	18.3	1.5	8.3%	8.4	21.0
Ash	9.7	0.3	3.4%	8.9	11.8
Carbohydrate	22.4	2.1	9.2%	18.0	30.1
P	1.4	0.1	3.7%	1.3	1.5
Energy (MJ/kg DM)	22.5	0.2	0.9%	22.1	23.0
Sum of Amino Acids	46.0	2.0	4.3%	40.9	49.6
ALA	2.7	0.1	4.6%	2.4	2.9
ARG	3.4	0.2	6.9%	2.9	3.9
ASP	4.7	0.4	7.5%	3.6	5.7
CYS	0.6	0.1	9.8%	0.5	0.8
GLU	7.8	0.4	5.2%	7.0	8.8
GLY	2.6	0.1	4.9%	2.3	2.8
HIS	1.3	0.1	9.9%	1.0	1.6
ISO	1.9	0.1	4.6%	1.7	2.1
LEU	3.7	0.2	4.7%	3.2	3.9
LYS	2.9	0.3	9.0%	2.3	3.3
MET	1.2	0.1	8.6%	1.1	1.5
PHE	2.0	0.1	3.6%	1.9	2.2
PRO	2.6	0.4	13.7%	1.9	3.5
SER	2.3	0.1	3.7%	2.1	2.5
TAU	0.3	0.0	9.0%	0.2	0.4
THR	2.1	0.1	4.8%	1.9	2.4
TYR	1.7	0.1	4.6%	1.5	1.8
VAL	2.0	0.1	5.8%	1.8	2.3
Diet digestibility coefficients					
Dry matter	0.732	0.018	2.4%	0.692	0.791
N	0.906	0.009	1.0%	0.881	0.923
Fat	0.956	0.017	1.7%	0.895	0.983
P	0.540	0.066	12.2%	0.382	0.664
Energy	0.810	0.016	1.9%	0.785	0.861
Sum of Amino Acids	0.932	0.008	0.9%	0.912	0.945

CV%: Coefficient of variation = SD / Mean x100

Table 7.4 Variability in ingredient composition across all test ingredients. All values are g/kg DM unless otherwise detailed.

	MEAN	SD	CV%	MIN	MAX
Dry matter (g/kg)	91.6	0.6	0.6%	90.4	92.8
Protein (N x 6.25)	45.4	3.4	7.6%	36.5	56.7
Fat	7.8	0.9	12.1%	5.2	9.7
Ash	3.0	0.4	14.0%	1.9	3.9
Carbohydrate	43.8	3.3	7.6%	32.7	53.9
P	0.4	0.1	15.3%	0.3	0.6
Energy (MJ/kg DM)	20.8	0.3	1.5%	20.1	21.5
Sum of Amino Acids	44.0	3.2	7.2%	33.2	53.7
ALA	1.6	0.1	6.8%	1.3	1.8
ARG	5.1	0.5	9.9%	4.0	6.6
ASP	4.9	0.4	7.7%	3.8	5.9
CYS	0.7	0.1	16.5%	0.5	1.3
GLU	10.0	0.8	7.8%	7.5	12.6
GLY	1.9	0.1	6.4%	1.5	2.1
HIS	1.1	0.1	11.8%	0.8	1.4
ISO	1.7	0.1	7.6%	1.3	2.0
LEU	3.2	0.3	8.0%	2.4	4.3
LYS	1.8	0.2	13.2%	1.2	2.4
MET	0.3	0.1	32.2%	0.2	0.7
PHE	1.8	0.2	12.4%	0.1	2.1
PRO	2.5	0.6	26.0%	1.0	4.3
SER	2.4	0.2	6.8%	1.9	2.9
THR	1.8	0.1	7.3%	1.5	2.1
TYR	1.7	0.2	9.1%	1.1	2.1
VAL	1.5	0.1	8.4%	1.2	1.8
Crude Fibre	30.9	4.6	14.9%	17.5	43.4
Neutral-Detergent Fibre	10.2	5.4	52.3%	5.2	26.2
Acid- Detergent Fibre	6.6	4.5	69.1%	3.0	20.0
Lignin	0.7	0.5	65.9%	0.2	2.2

CV%: Coefficient of variation = SD / Mean x100

Table 7.5 Variability in ingredient digestibility parameters and digestible values across all test ingredients.

	MEAN	SD	CV%	MIN	MAX
Ingredient digestibility coefficients					
Dry matter	0.532	0.050	9.5%	0.391	0.655
N	0.933	0.096	10.3%	0.655	1.146
Fat	0.735	0.593	80.7%	-3.151	1.818
P	1.834	0.884	48.2%	0.126	3.970
Energy	0.573	0.046	8.0%	0.482	0.694
Sum of Amino Acids	0.880	0.130	14.8%	0.526	1.265
Digestible value (% dry basis)					
Dry matter	48.7	4.7	9.6%	35.8	59.8
Protein (N x 6.25)	42.3	4.8	11.3%	30.4	54.7
Fat	5.9	3.3	55.1%	0.0	9.7
P	0.7	0.3	44.0%	0.1	0.6
Energy (MJ/kg dry basis)	11.9	1.0	8.2%	9.9	14.5
Sum of Amino Acids	38.7	6.2	16.1%	23.4	50.6

CV%: Coefficient of variation = SD / Mean x100.

Table 7.6 Correlation matrices among diet digestibility parameters and diet compositional parameters from the experimental diets (n=76).

Diet Constituent	Protein	Fat	ProFat	CHO	Energy	sAA
Diet ADC-N	-0.0220 p=0.844	0.3170 p=0.004	0.2123 p=0.055	0.1927 p=0.083	-0.1939 p=0.081	-0.2598 p=0.018
Diet ADC-Energy	0.161 p=0.148	0.3488 p=0.001	0.4105 p=0.000	-0.2691 p=0.014	-0.0957 p=0.393	-0.0708 p=0.527
Diet ADC-sAA	-0.0806 p=0.471	0.3059 p=0.005	0.1482 p=0.184	0.1422 p=0.202	-0.1568 p=0.159	-0.1088 p=0.330

ProFat: Protein + Fat. CHO: Carbohydrate. SAA: sum of Amino acids.

Table 7.7 Correlation matrices among ingredient digestibility parameters and ingredient compositional parameters from the experimental kernel meals (n=76), for parameters of crude fibre, ADF, NDF and Lignin, n=35.

Ingredient Constituent	Apparent Digestibility Coefficient		
	Nitrogen	Energy	sum Amino Acids
Protein	-0.2946 p=.086	0.4659 p=.005	0.2624 p=.128
Fat	0.5148 p=.002	-0.0815 p=.642	-0.2523 p=.144
ProFat	-0.1765 p=.310	0.4738 p=.004	0.2120 p=.221
CHO	0.2055 p=.236	-0.4904 p=.003	-0.2460 p=.154
Energy	0.2130 p=.219	0.2343 p=.176	0.0629 p=.720
sAA	-0.1863 p=.284	0.5694 p=.000	0.0255 p=.884
Fibre	0.2088 p=.229	-0.1950 p=.262	-0.0589 p=.737
NDF	-0.0004 p=.998	-0.0072 p=.967	0.0641 p=.714
ADF	-0.0812 p=.643	-0.0263 p=.881	0.0408 p=.816
Lignin	-0.7036 p=.000	0.1302 p=.456	0.0201 p=.909

ProFat: Protein + Fat. CHO: Carbohydrate. SAA: sum of Amino acids.

Table 7.8 Correlation matrices among ingredient digestible values and ingredient compositional parameters from the experimental kernel meals (n=76), for parameters of crude fibre, ADF, NDF and Lignin, n=35.

Ingredient Constituent	Digestible value Protein	Energy	sum Amino Acids
Protein	0.4109 p=.014	0.4978 p=.002	0.7197 p=.000
Fat	0.2770 p=.107	0.0280 p=.873	-0.2718 p=.114
ProFat	0.5106 p=.002	0.5368 p=.001	0.6931 p=.000
CHO	-0.4921 p=.003	-0.5421 p=.001	-0.7170 p=.000
Energy	0.4836 p=.003	0.4164 p=.013	0.3066 p=.073
sAA	0.4372 p=.009	0.6192 p=.000	0.5801 p=.000
Fibre	-0.1207 p=.490	-0.2000 p=.249	-0.3071 p=.073
NDF	-0.0556 p=.751	-0.0066 p=.970	-0.0649 p=.711
ADF	-0.1254 p=.473	-0.0304 p=.862	-0.0742 p=.672
Lignin	-0.4981 p=.002	0.0505 p=.773	0.1528 p=.381

ProFat: Protein + Fat. CHO: Carbohydrate. SAA: sum of Amino acids.

Table 7.9 Multiple regression analyses of ingredient digestible values, ingredient protein content and additional compositional parameters from the experimental kernel meals (n=76), for parameters of crude fibre, ADF, NDF and Lignin, n=35.

Ingredient Constituents	Digestible value Protein	Energy	sum Amino Acids
Protein and Fat	R = 0.5552 p = 0.0000	R = 0.3847 p = 0.0018	R = 0.6008 p = 0.0000
Protein and CHO	R = 0.5340 p = 0.0000	R = 0.3708 p = 0.0029	R = 0.6079 p = 0.0000
Protein and Energy	R = 0.5469 p = 0.0000	R = 0.3886 p = 0.0016	R = 0.6421 p = 0.0000
Protein and sAA	R = 0.5511 p = 0.0000	R = 0.4072 p = 0.0008	R = 0.6038 p = 0.0000
Protein and Fibre	R = 0.4233 p = 0.0425	R = 0.5015 p = 0.0097	R = 0.7228 p = 0.0000
Protein and NDF	R = 0.4114 p = 0.0515	R = 0.5051 p = 0.0090	R = 0.7229 p = 0.0000
Protein and ADF	R = 0.4152 p = 0.0484	R = 0.5004 p = 0.0099	R = 0.7210 p = 0.0000
Protein and Lignin	R = 0.7693 p = 0.0000	R = 0.5082 p = 0.0084	R = 0.7226 p = 0.0000

Those relationships $p < 0.001$ are indicated in red.

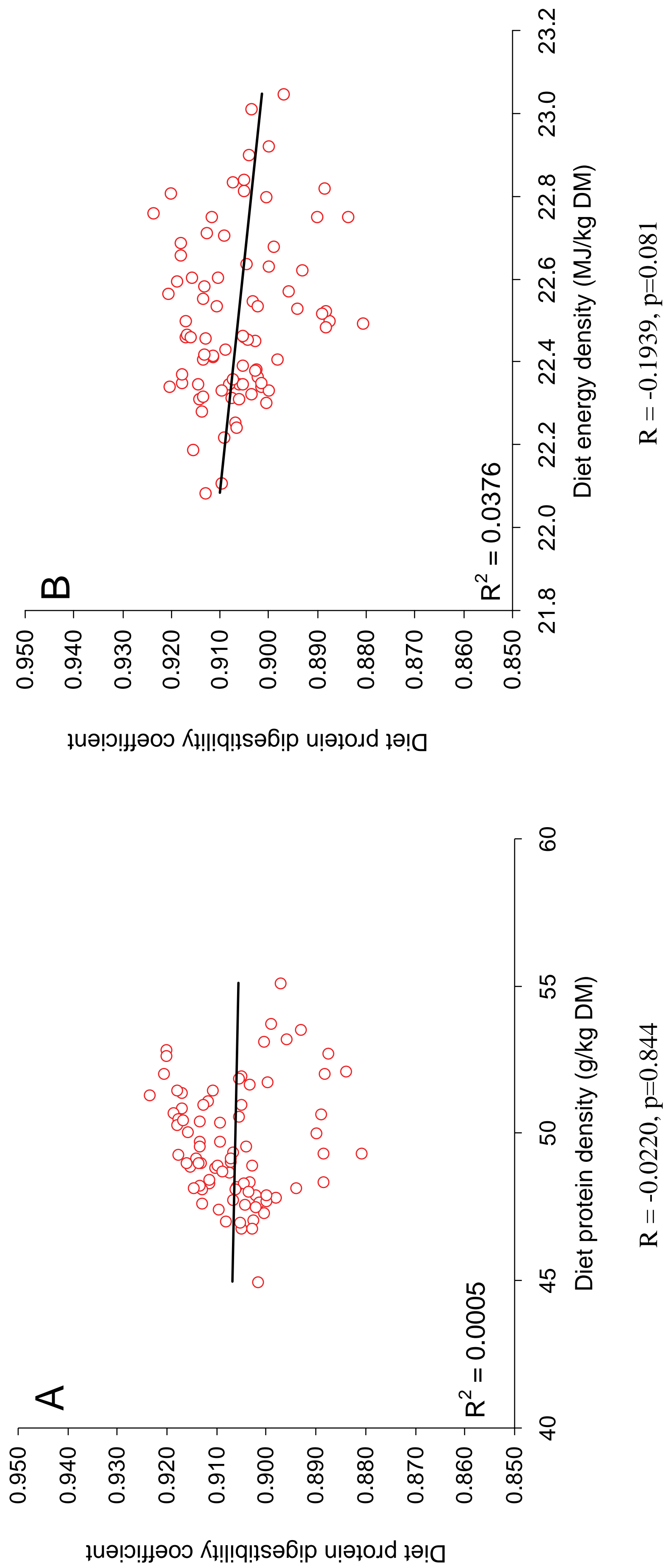


Figure 7.1 Influence of diet protein density (A), energy density (B) composition on apparent protein digestibility coefficients of the experimental diets.

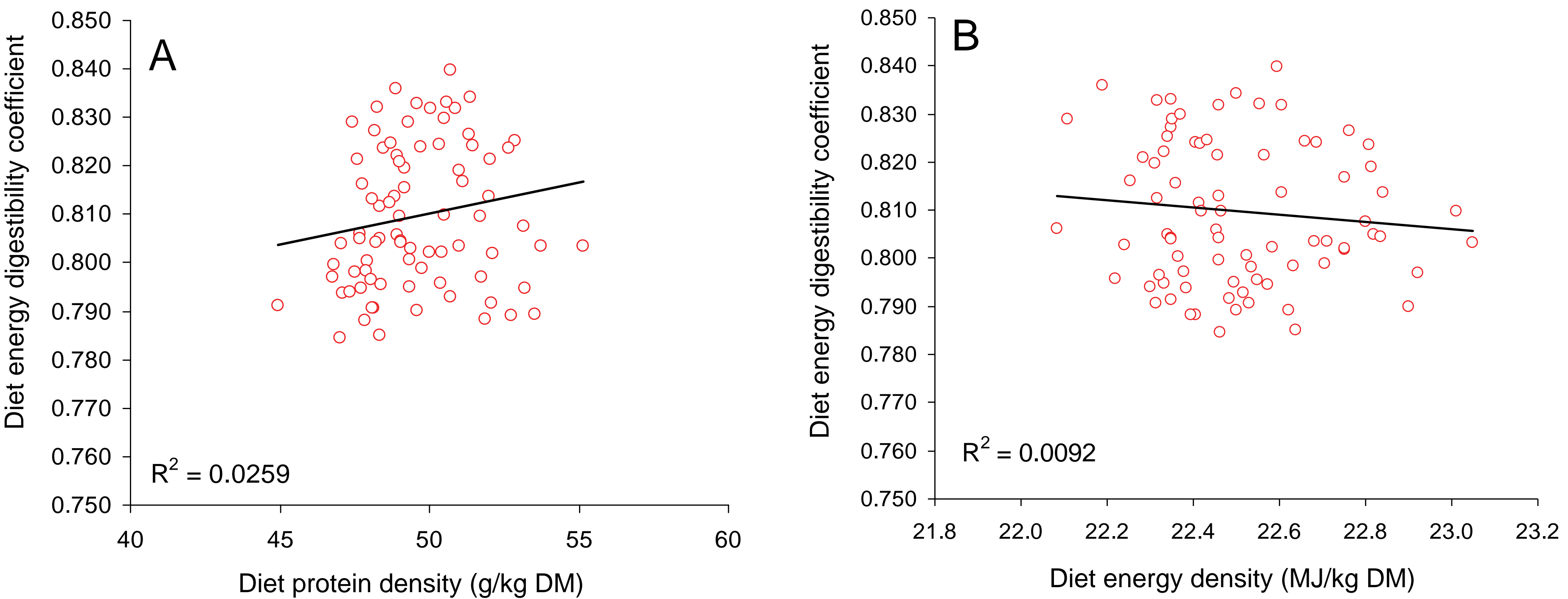


Figure 7.2 Influence of diet protein density (A) and energy density (B) on apparent energy digestibility coefficients of the experimental diets.

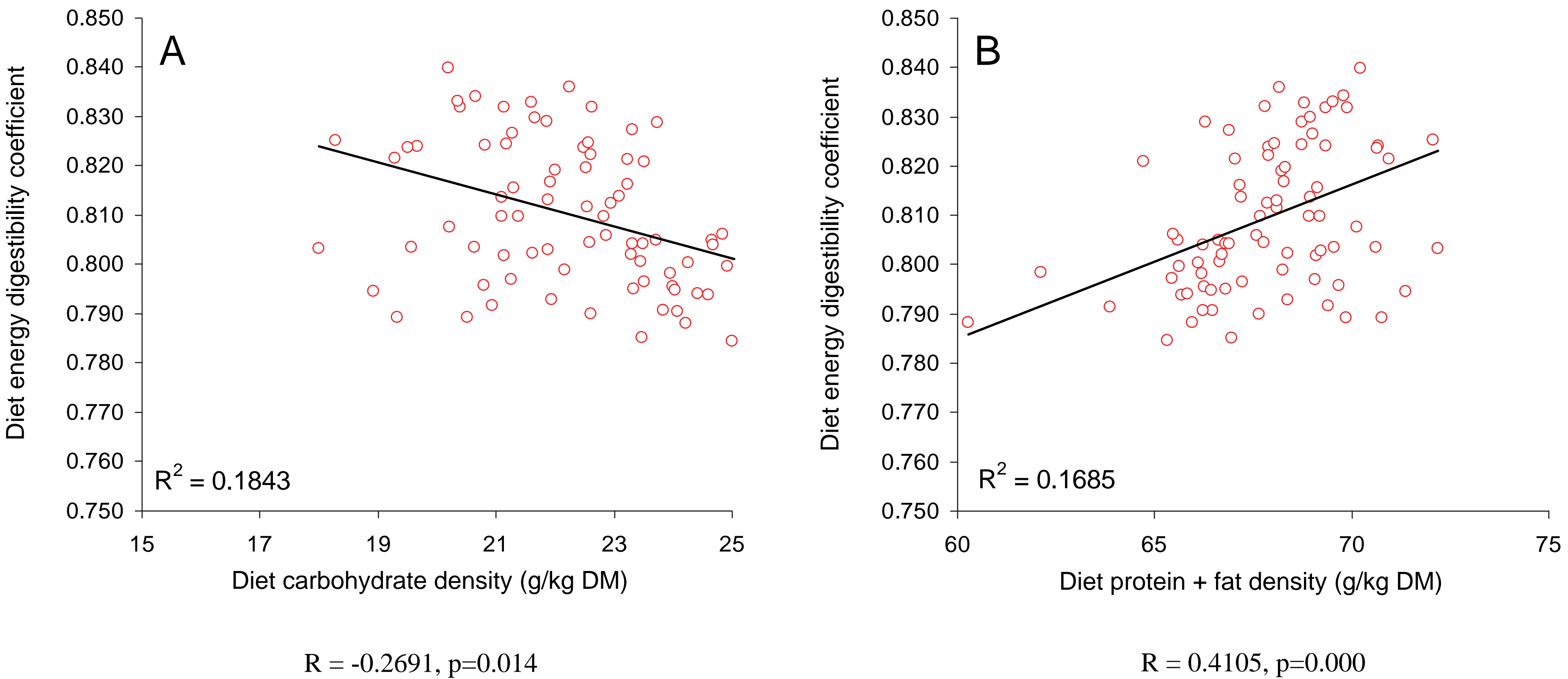


Figure 7.3 Influence of diet carbohydrate density (A), and protein + fat density (B) on apparent energy digestibility coefficients of the experimental diets.

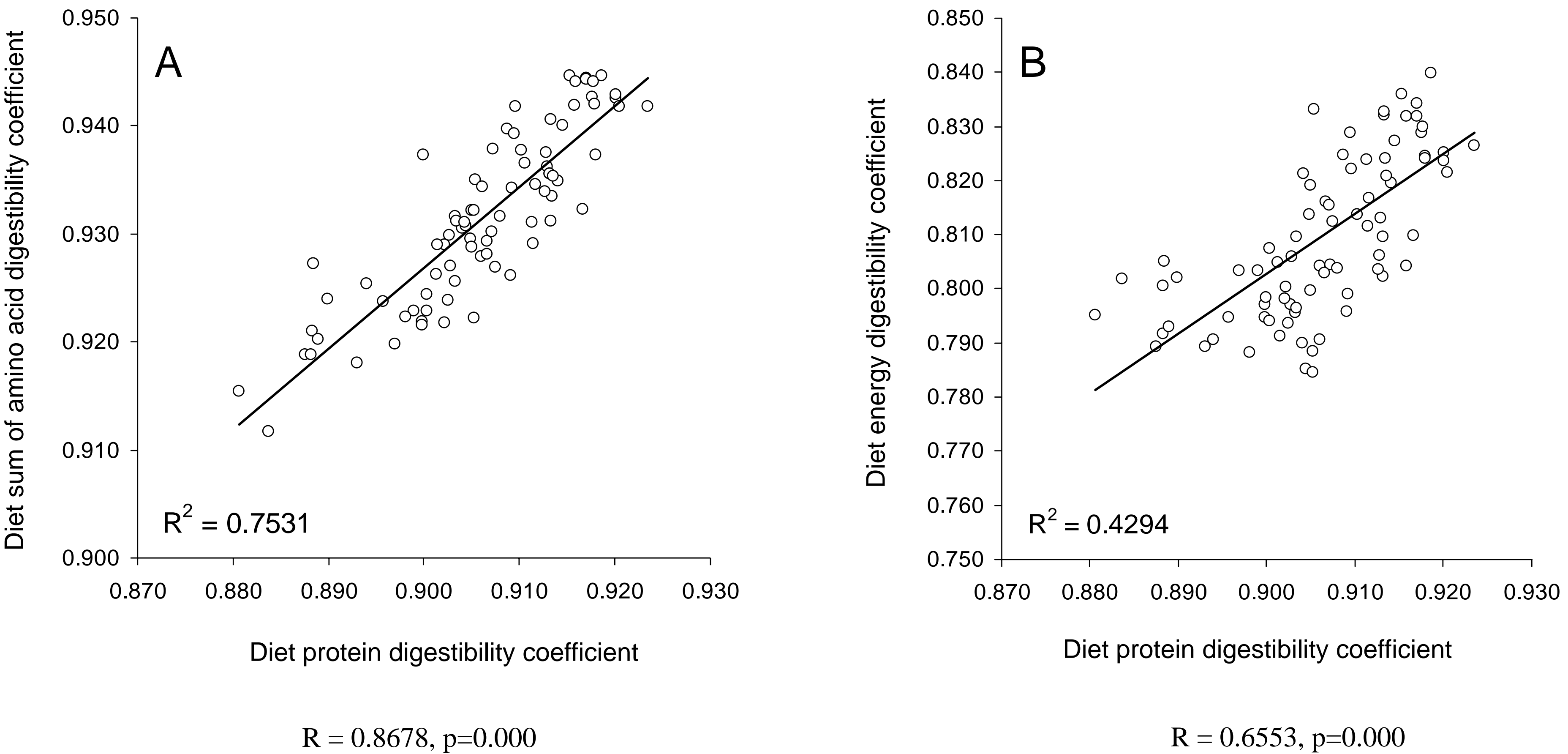


Figure 7.4 Relationships between protein digestibility and sum of amino acids (A) or energy (B) within the experimental diets.

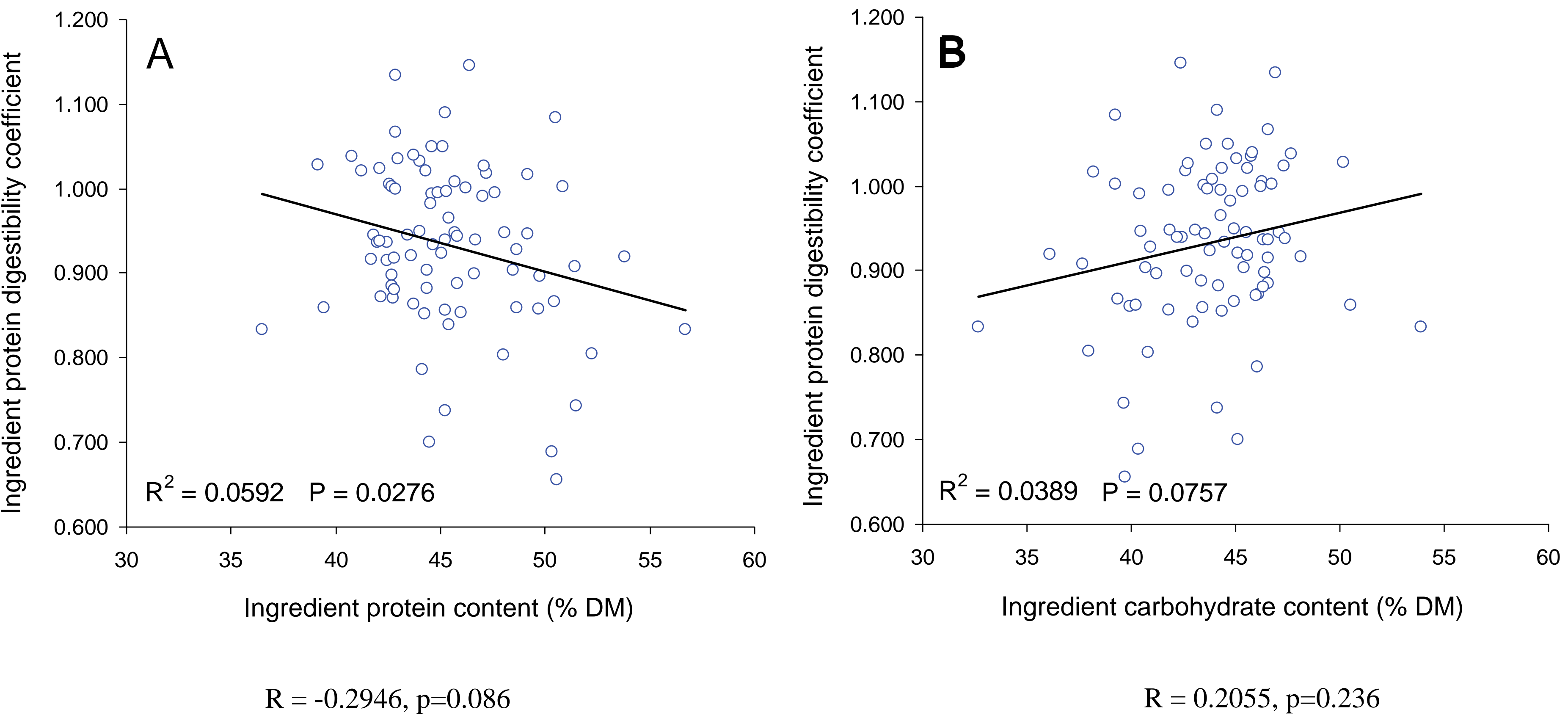


Figure 7.5 Influence of lupin kernel meal protein content (A) or carbohydrate content (B) on protein (nitrogen) digestibility of the lupin kernel meals.

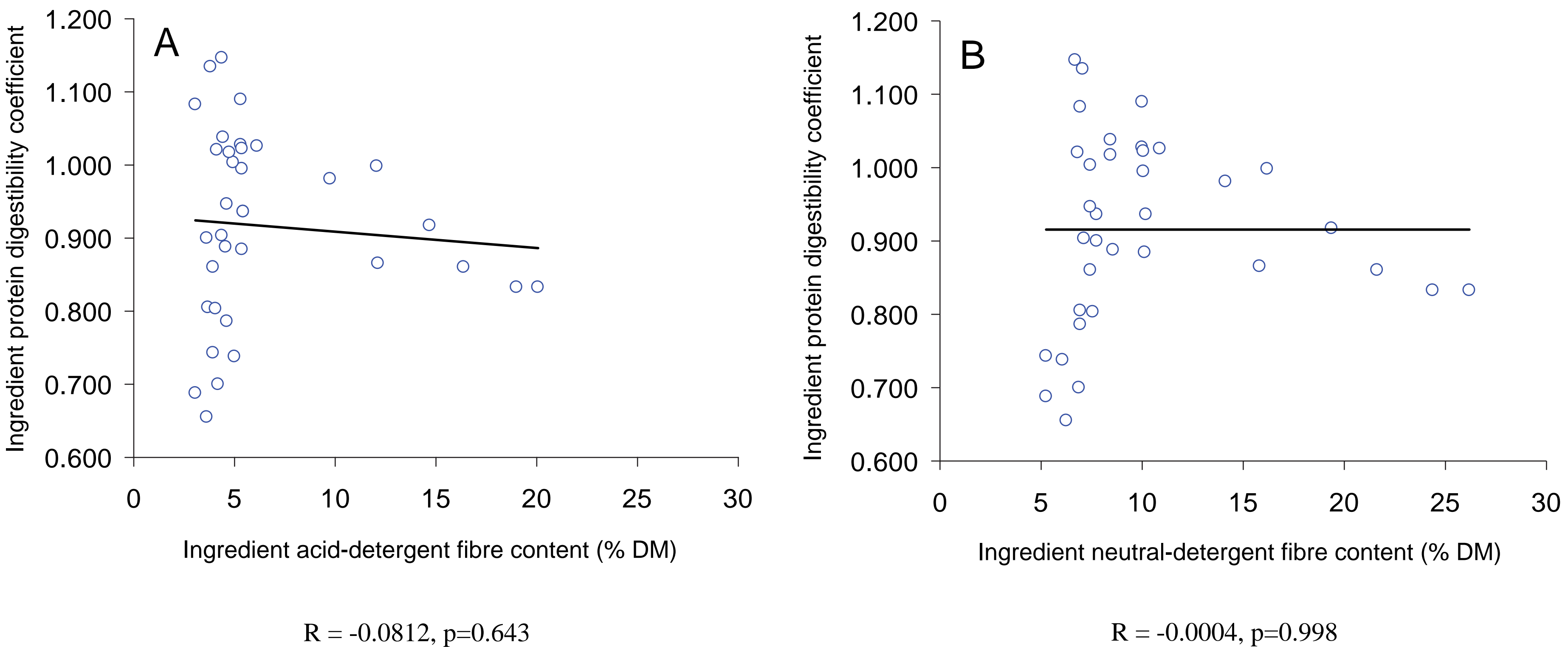
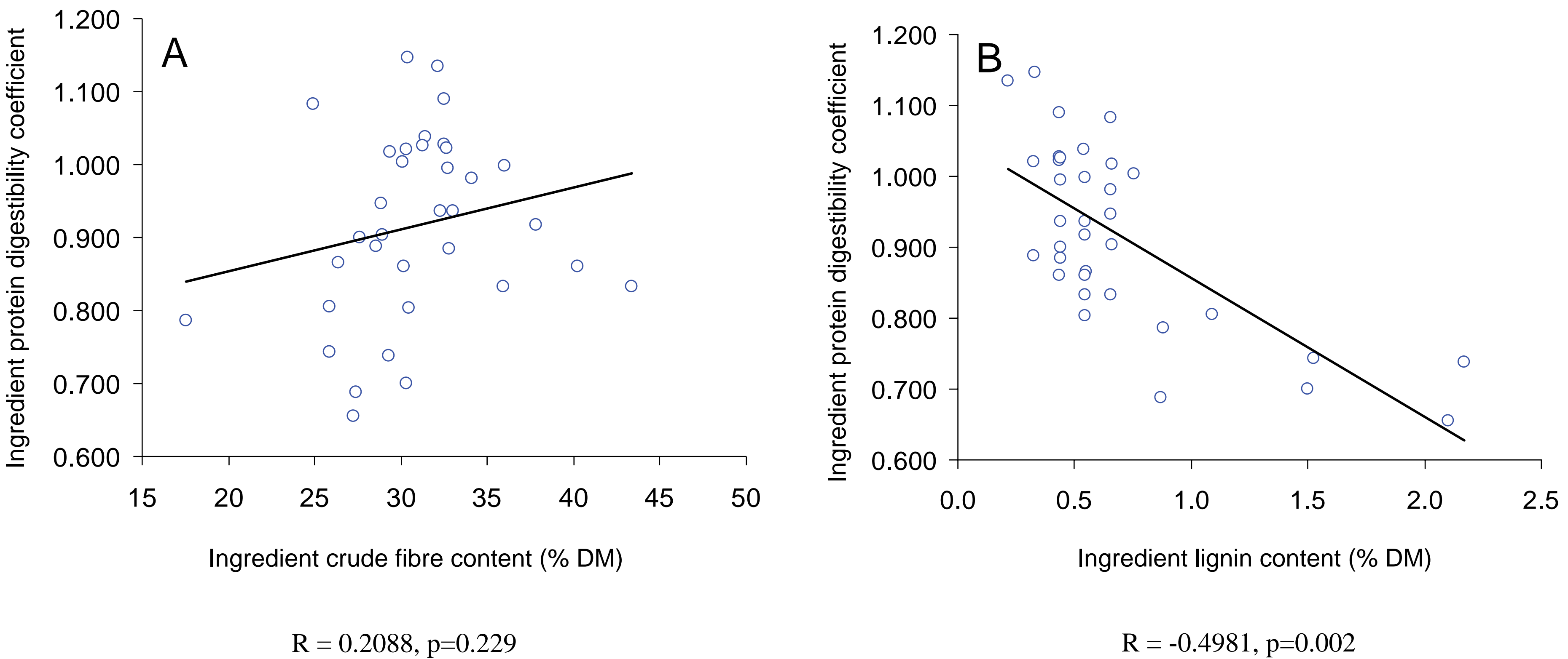


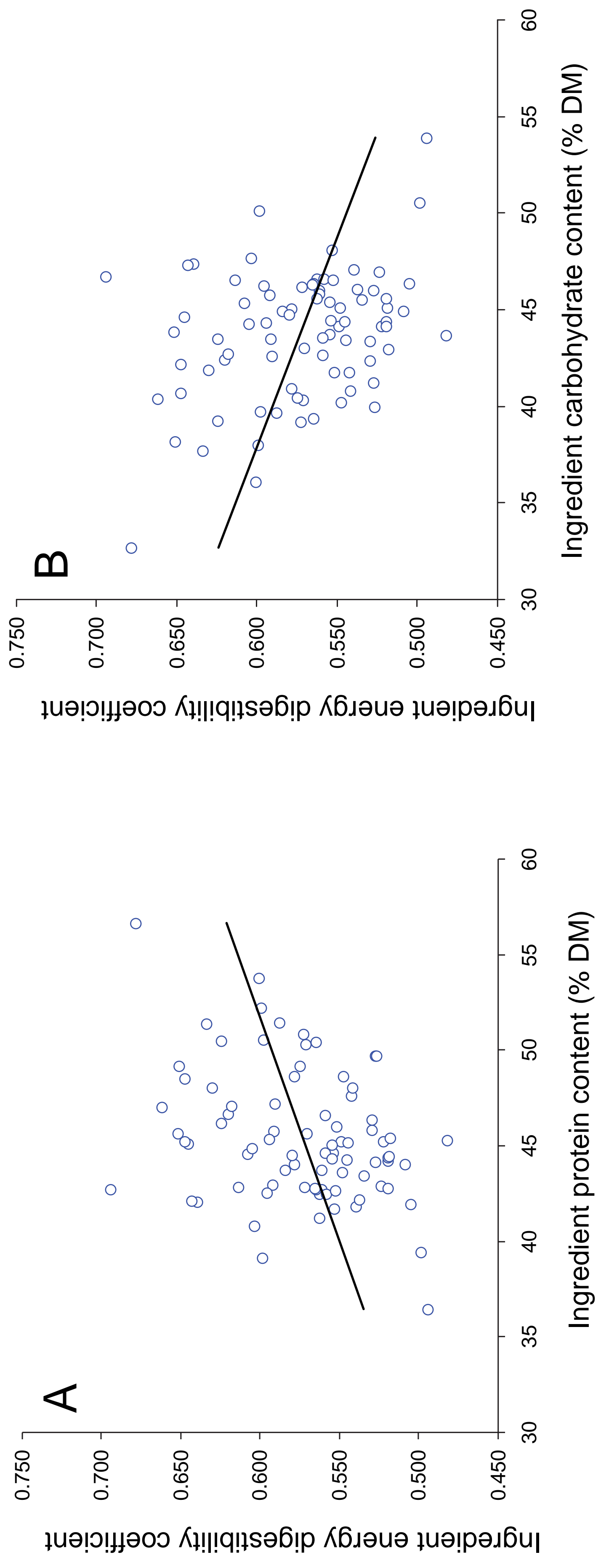
Figure 7.6 Influence of lupin kernel meal acid-detergent fibre content (A) or neutral-detergent fibre content (B) on protein digestibility of the lupin kernel meals.



R = 0.2088, p=0.229

R = -0.4981, p=0.002

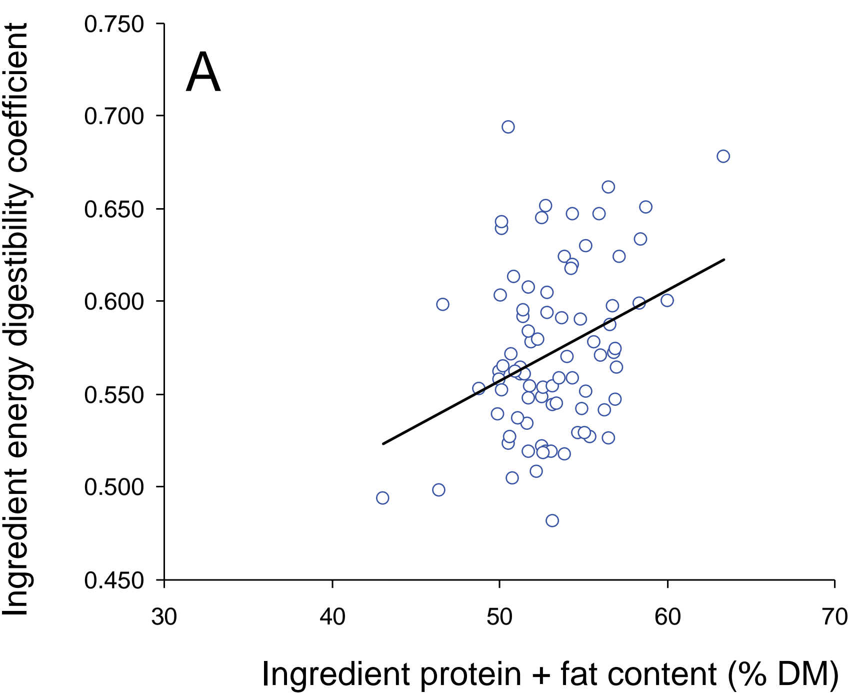
Figure 7.7 Influence of lupin kernel meal crude fibre content (A) or lignin content (B) on protein digestibility of the lupin kernel meals.



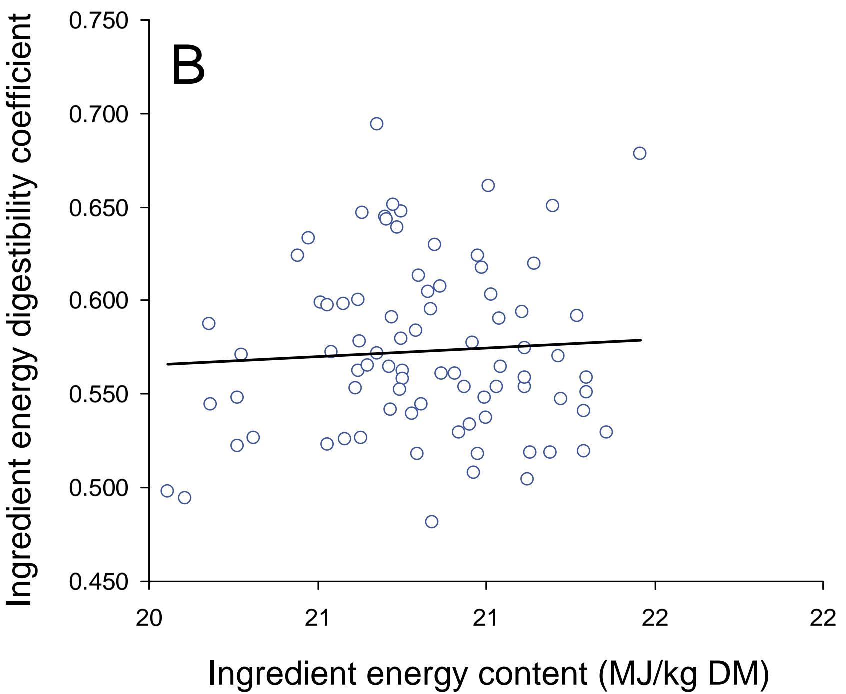
R = 0.4659, p=0.005

R = -0.4904, p=0.003

Figure 7.8 Influence of lupin kernel meal protein (A) and carbohydrate (B) content on the energy digestibility of lupin kernel meals.



R = 0.4738, p=0.004



R = 0.2343, p=0.176

Figure 7.9 Influence of lupin kernel meal protein + fat (A), or gross energy (B) content on the energy digestibility of lupin kernel meals.

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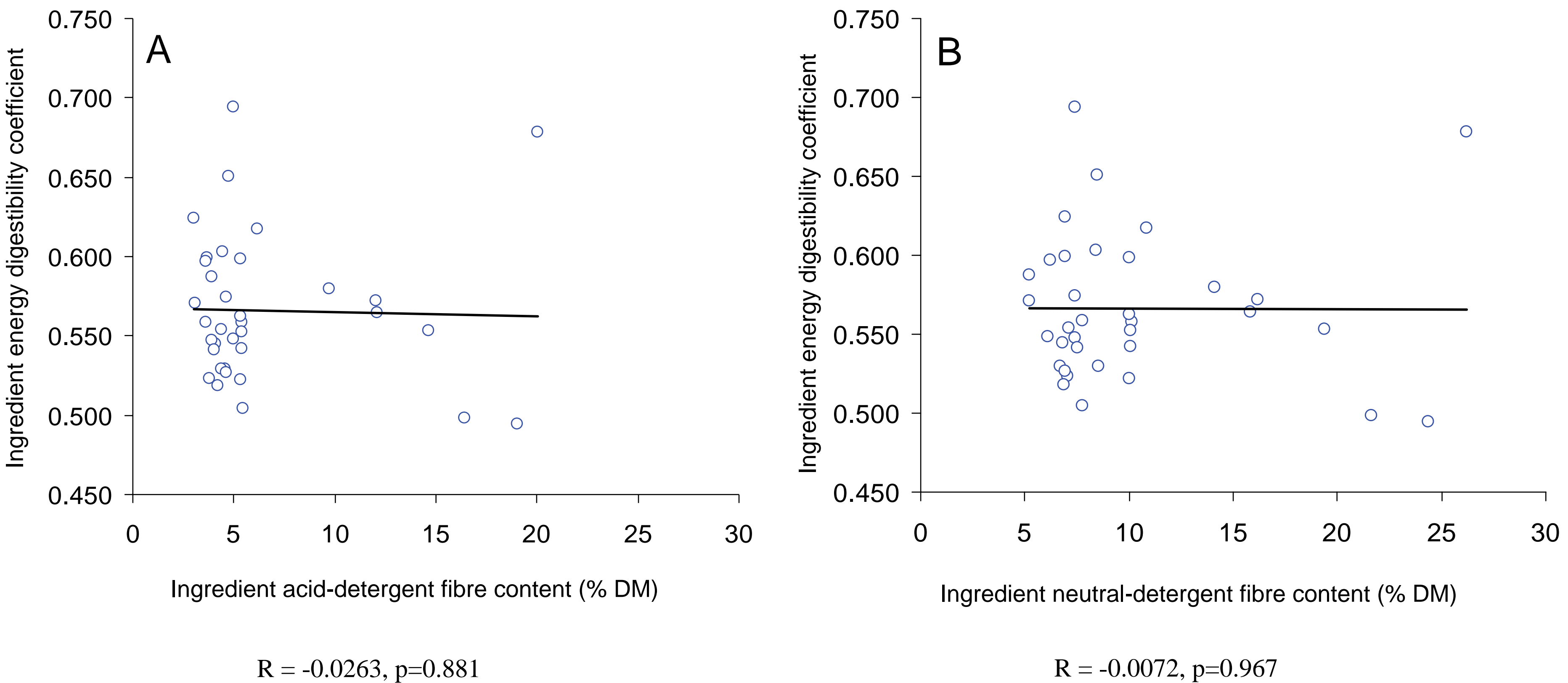


Figure 7.10 Influence of lupin kernel meal acid-detergent fibre content (A) or neutral-detergent fibre content (B) on energy digestibility of the lupin kernel meals.

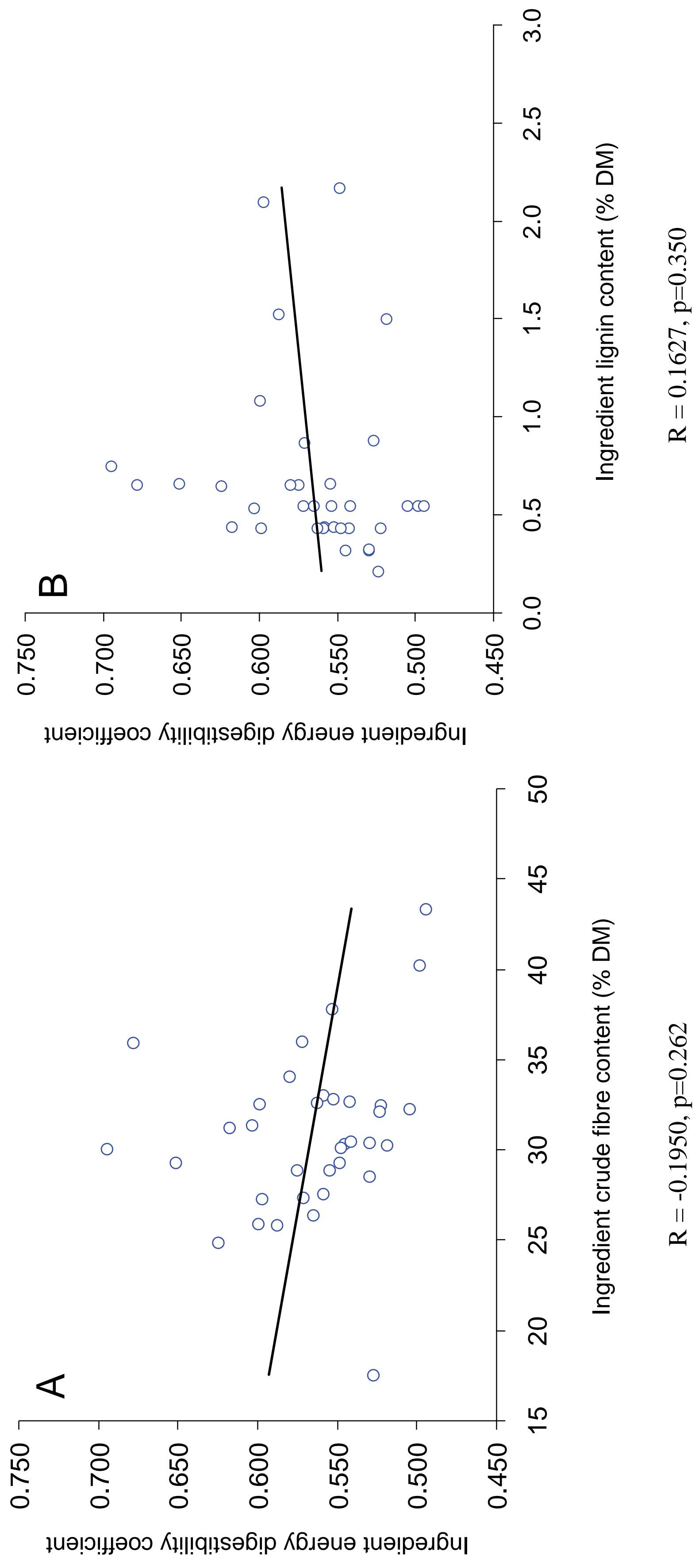


Figure 7.11 Influence of lupin kernel meal crude fibre content (A) or lignin content (B) on energy digestibility of the lupin kernel meals.

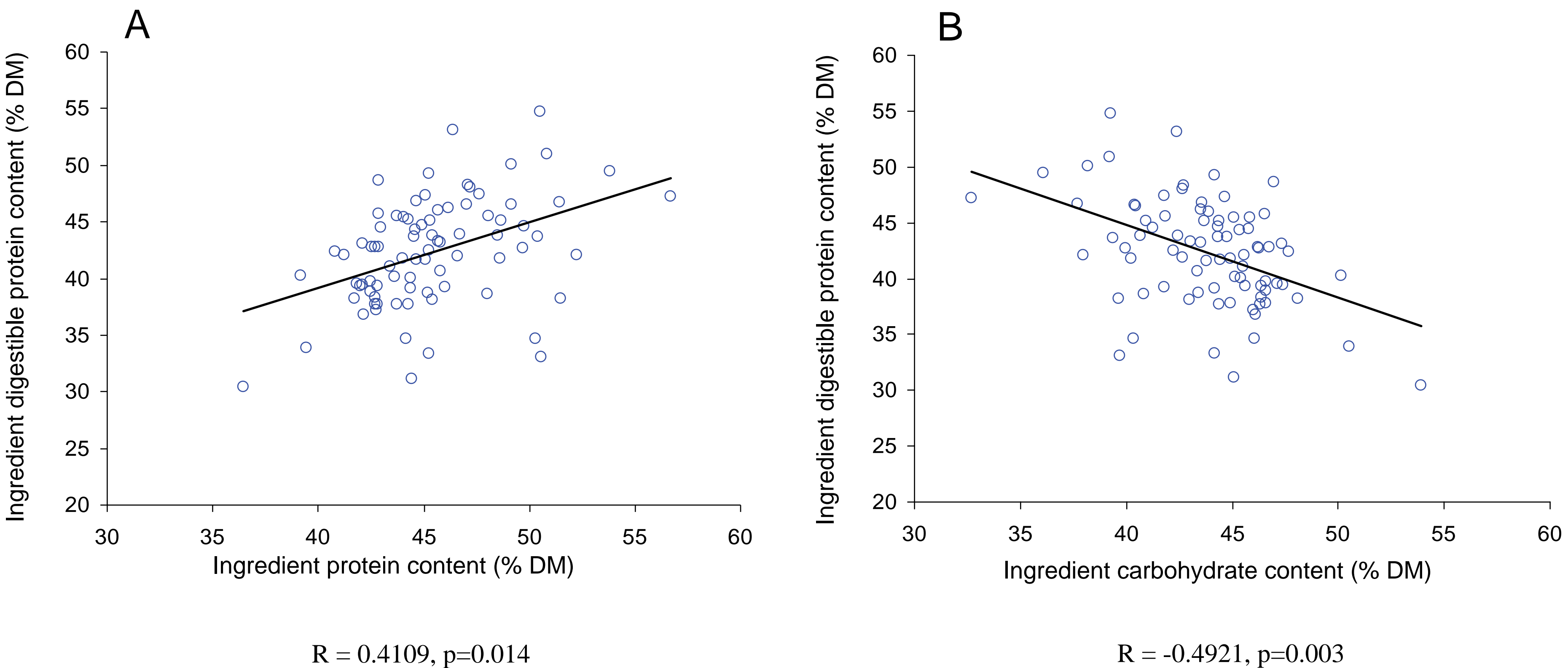
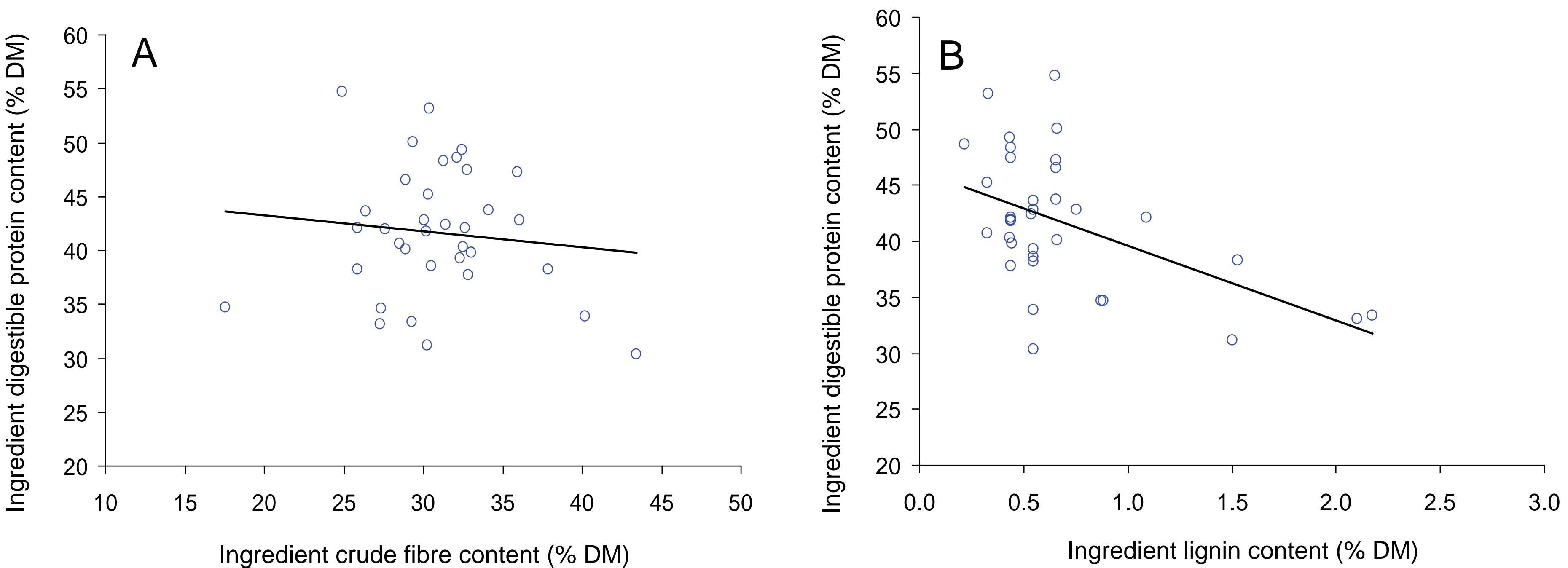


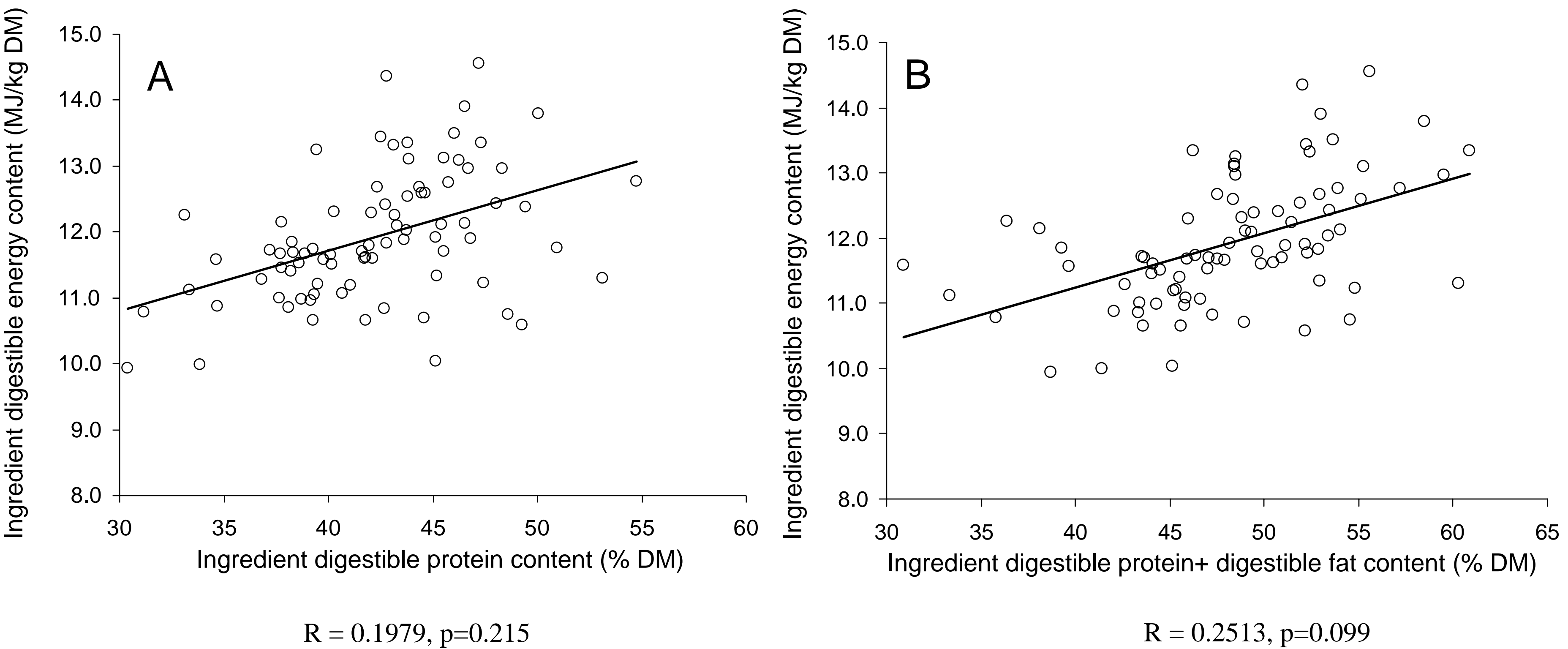
Figure 7.12 Influence of lupin kernel meal crude protein (A) or crude carbohydrate (B) levels on the digestible protein content of lupin kernel meal.



R = -0.1207, p=0.490

R = -0.7036, p=0.000

Figure 7.13 Influence of lupin kernel meal crude fibre (A) or lignin (B) levels on the digestible protein content of lupin kernel meal.



R = 0.1979, p=0.215

R = 0.2513, p=0.099

Figure 7.14 Influence of lupin kernel meal digestible protein content (A) or digestible protein + digestible fat content (B) on the digestible energy content of each lupin kernel meal.

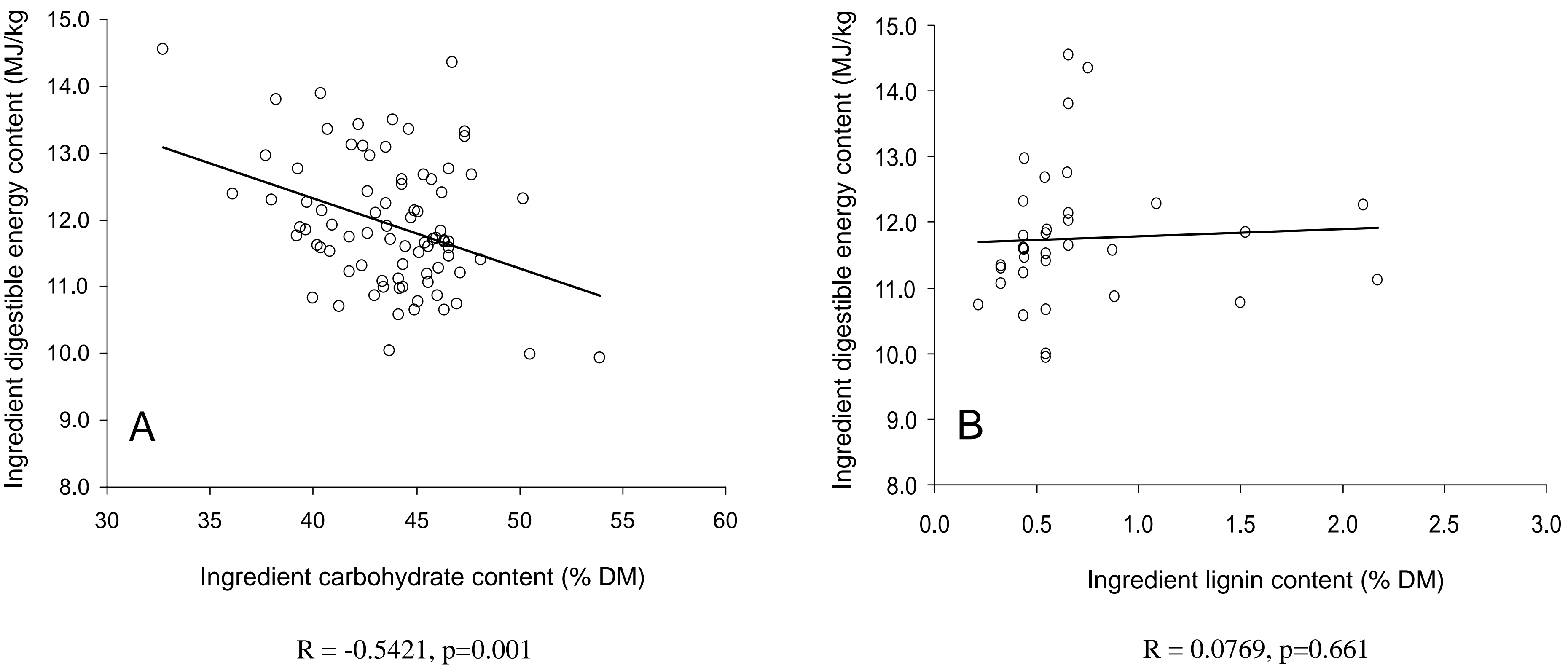


Figure 7.15 Influence of lupin kernel meal crude carbohydrate on the digestible energy content of each lupin kernel meal.

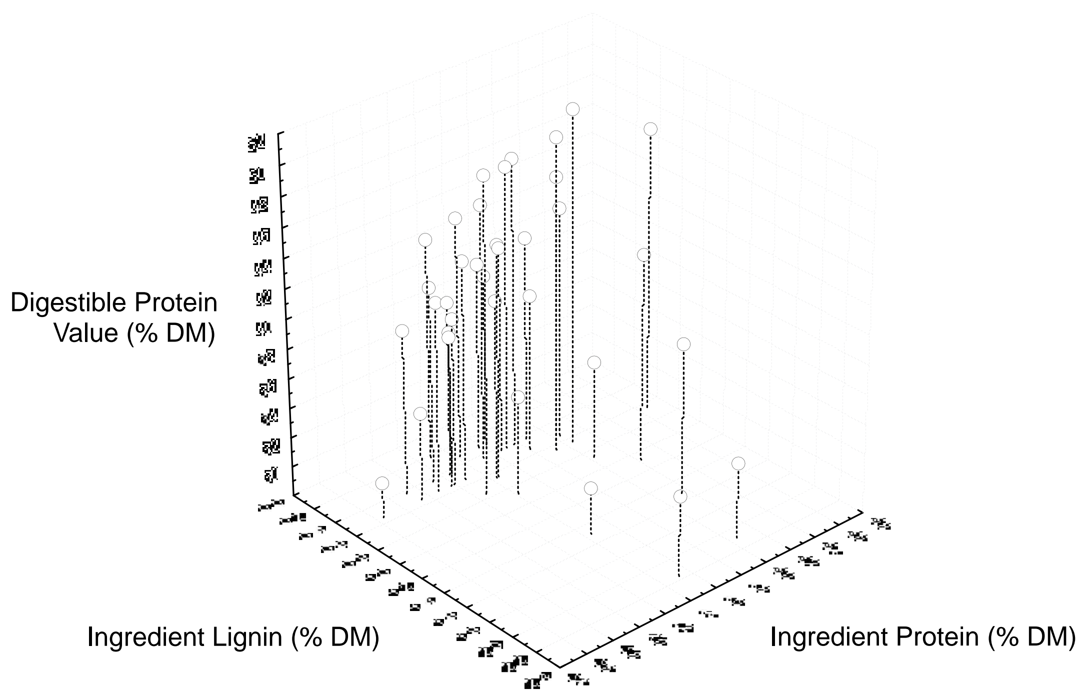


Figure 7.16 Dual influence of lupin kernel meal protein and lignin on the digestible protein content of each lupin kernel meal.

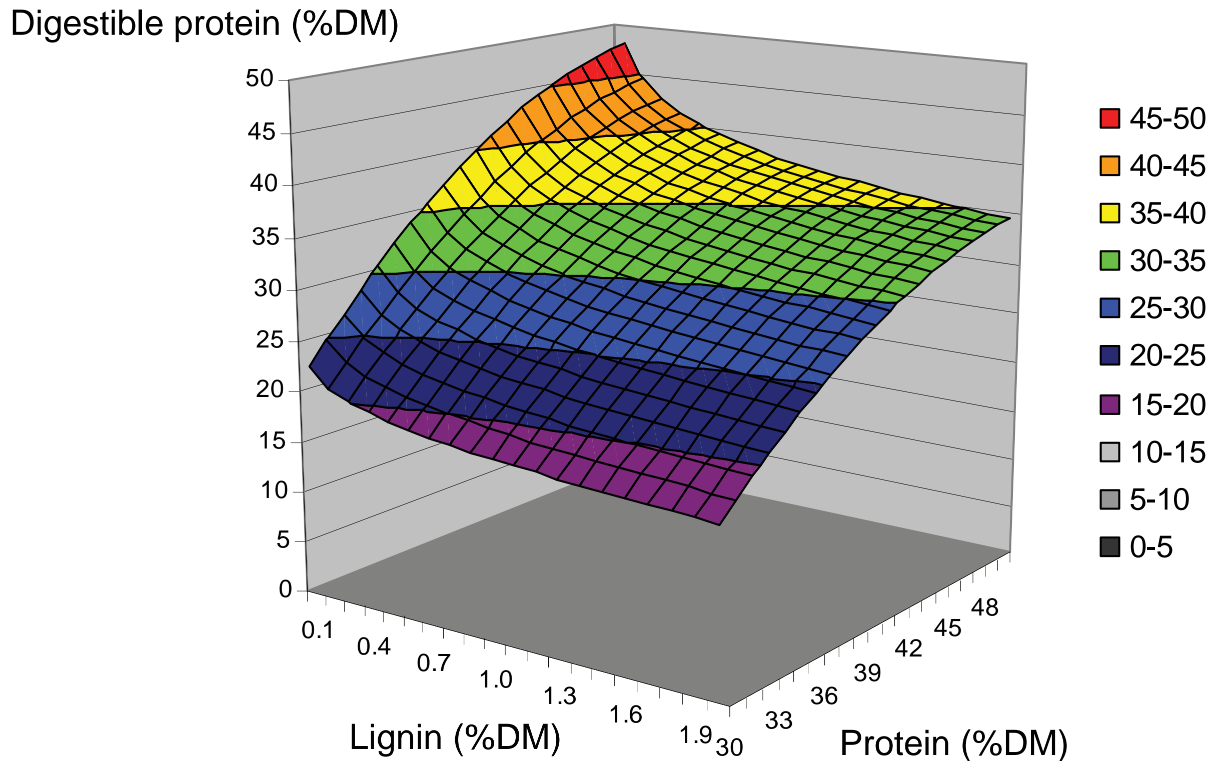


Figure 7.17 Model of the dual influence of lupin kernel meal protein (%DM) and lignin (%DM) on the digestible protein content (%DM) of lupin kernel meal when fed to rainbow trout.

8.0 Evaluating Near Infrared Reflectance Spectroscopy (NIRS) to predict the nutrient composition, energy value and digestibility of lupin kernel meals when fed to rainbow trout, *Oncorhynchus mykiss*

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Abstract

This study examined the ability of NIRS to predict nutrient composition, energy value and digestibility of lupin kernel meals when fed to rainbow trout by scanning both whole lupin seeds and kernel meal. Kernel meal samples of narrow-leaf lupins, *Lupinus angustifolius* that were to be used to prepare diets for rainbow trout feeding trials were scanned using a Bruker MPA Fourier transform near infra red (FTNIR) spectrophotometer (the whole seeds were also scanned where they were available.). After the chemical analyses were completed on the constituent kernel meal samples, and the diets, the digestibility of both were also evaluated by analysing the fish faeces. The NIRS spectra were then used to create calibrations (regression equations) using the OPUS® chemo-metrics software package. This exercise involved running optimisation experiments to find the best math pre-treatment and wavelength segment(s) for each digestibility, nutrient and energy data set. In some cases values were excluded as outliers to the regression. The results are reported in terms of standard error of cross validation (SECV) and correlation coefficient (R^2). Cross-correlation between the predicted values was also evaluated and compared to that of the raw data. Viable calibrations were obtained for Protein, Protein plus Fat, Carbohydrate and the Sum of the Amino Acids. These parameters all had SECVs less than or equal to the standard error of the reference method and no greater than half the standard deviation of the population under consideration. Several other parameters were close to being acceptable lacking only a larger variation in the population relative to their SECVs. The findings of the study indicate the potential to use NIRS to rapidly and non-destructively evaluate the nutrient composition and energy of lupin meal used in fish diets and even to predict the digestibility of some of these values.

8.1 Introduction

When preparing aquaculture diets containing lupin kernel flour it is desirable to be able to evaluate the nutrient composition and energy value of these ingredients as well their likely digestibility as a component of the diet. NIRS provides the opportunity to rapidly and non-destructively predict these values just prior to diet preparation. This study reports on the ability of NIRS to predict nutrient composition, energy value and digestibility of narrow-leaf lupin, *Lupinus angustifolius* kernel meals when fed to rainbow trout by scanning both whole lupin seeds and kernel meal. The parameters covered by this study include the Dry Matter (*I-DM*), Protein (*I-Protein*), Protein plus Fat (*I-ProFat*), Carbohydrate (*I-CHO*), Energy (*I-Energy*), and

the Sum of the Amino Acids (*I-sAA*) of the kernel flour ingredients, and, their digestible value for Protein (*DV-Pro*), Energy (*DV-E*), and the Sum of Amino Acids (*DV-sAA*). Inter-correlations between the nutrient composition, energy value and digestibility values were examined to find what inter-correlations exist between the data sets. The NIRS was used to obtain spectra from both whole seed and kernel flour samples prior to their use as ingredients in the production of diets used in rainbow trout feeding trials. Chemo-metrics software was used to process the NIR spectra to produce prediction equations for the nutrient composition, energy and digestibility values. These predictions were evaluated in relation to the inherent variability involved in the study. The calibration statistics were obtained from both kernel meal and whole seed spectra but only the kernel meal data is reported here.

8.2 Materials and Methods

8.2.1 Lupin kernel meal production, digestibility and chemical analysis

Over a three-year period, separate batches of seed of *Lupinus angustifolius* were collected from the Department of Agriculture's (WA) germ-plasm and breeding lines. This seed in many cases constituted the same genotype over several seasons, often from the same site. In each case one sample of the seeds were then dehulled using a small disc-mill and aspirated to separate hulls from kernels. (If sufficient sample was available whole seed was also kept for NIRS). A final manual clean of the kernel samples was done to remove any remaining hull material to ensure purity of the kernel preparation. The kernel samples were then milled using a Retsch Hammermill with a 750 µm screen to create samples of kernel flour. In addition to the lupin kernel flours each of the test ingredients used in this study was thoroughly ground such that they passed through a 750 µm hammer mill screen. These kernel flour samples were used to formulate diets for the rainbow trout feeding/digestibility trials as described previously (Glencross et al., 2005) and sub-samples were sent for analysis. Faeces from the feeding trial for digestibility analysis were collected using stripping techniques (Glencross et al., 2005). These samples were stored to prevent contamination in a freezer at -20°C before being freeze-dried in preparation for analysis. Triplicate samples were analysed for each digestibility variable with the mean value used in this study.

8.2.2 Analysis of variability and inter-correlation of digestibility, nutrient and energy value data

To assess the variation in the ingredient and digestibility data 7 replicates of a reference sample of *Lupinus angustifolius* (cv Myallie) were included in the study. This provided information about the error background involved in the sampling and analytical techniques involved that is critical to understanding the effectiveness of NIRS calibrations. The variation in nutrient and energy measurement in terms of the mean and standard deviation of the values for the reference sample, as well as the range and standard deviation for the whole population is shown in Table 8.1. The same information for the digestibility data is given in Table 8.2. This data is essential for establishing the NIRS calibrations since the SECVs generated can only be validated by comparison to the background errors of the experiment.

As part of the evaluation of the NIRS calibrations the inter-correlation of prediction values must be examined to ensure that the regressions used are independently derived. In practice some inter-correlation is inevitable since the same spectra are being used and there are inherent relationships in the reference data. (A detailed analysis of the influence of ingredient composition on the digestibility

is provided in the paper cited above). Thus as a prelude to the evaluation of NIRS prediction models the inter-relationships between the parameters of interest in this study were examined.

8.2.3 NIRS scanning of *Lupinus angustifolius* seed and kernel flour samples

A Bruker Fourier Transform MPA and the OPUS® software package (Ver 5.5, © 2004 Bruker Optik GmbH, Rudolf-Plank-Straße 27, D-76275, Ettlingen) was used to scan 74 kernel flour (and 44 seed) samples in duplicate. These samples were scanned in a temperature controlled atmosphere with the instrument operated in reflectance mode using the rotating 97 mm sample cup. The spectra from the samples were collected across the full Wave Number range (12,493 to 3,599 cm^{-1}) of the instrument as absorbance with a bandwidth of 8 cm^{-1} using 64 scans per sample. The full set of kernel meal spectra in the Wave Number range used by many of the calibrations is shown in Figure 8.1.

8.2.4 Chemo-metrical analysis

Initially the individual spectra were examined visually to eliminate the possibility of any anomalous scans before they were incorporated into the OPUS® QUANT multi-variate calibration software (©Bruker Optik, as above). The reference data was then copied into Opus® to form the calibration data set. The spectra were evaluated as the mean of two scans. The OPUS® optimisation program incorporating a partial least square (PLS) fit method was then used to develop calibration models. This produced regression equations based on selected parts of the spectra after specific mathematical treatments of the data. Cross validation tests were then run for each parameter in turn using the suggested calibration models that incorporated appropriate Wave Number ranges and math pre-treatments. The calibrations were evaluated by examining the statistical measurements of the standard error of cross validation (SECV) and the correlation coefficient (R^2). The SECV is the standard deviation of differences between the reference values and values calculated by the regression equation when leaving out each sample in turn and using the rest of the population in the model to predict it. The validation tests were usually run several times after excluding outliers (samples the software flags as either bad reference results or extremely unusual spectrally). This process was continued until a balance was struck that included the following elements.

- The standard error of cross validation (SECV) is similar to the standard error of the reference method.
- The number of outliers (poor prediction samples) is small enough or their residual vales are low enough to still be able to meet the objectives of the calibration.
- The correlation coefficient (R^2) is sufficiently close to a perfect correlation of 1.0 to indicate probable future robustness and to meet the objectives of the calibration.

Also depending on the purpose of the measurements, an NIRS calibration is usually only viable if the SECV value is similar to the standard error of the reference method and is no more than a half (preferably a third or less) of the standard deviation of the data set used to produce the calibration (or future prediction population). R^2 values of 0.6 or even lower can be acceptable in a NIRS calibration, although values of over 0.8 are desirable for calibration robustness. The results need also to be examined for cross-correlation to ensure the NIRS calibrations are not merely mirroring each other as a result of a common spectral relationship. Some cross-correlations do of course occur naturally, such as the inverse relationship between protein and carbohydrate in most grain legumes including lupins.

8.3 Results

8.3.1 Ingredient composition and energy value calibrations

Viable calibrations were produced for *I-Protein*, *I-ProFat*, *I-CHO* and *I-sAA* (See Table 8.1). The DM and Energy data however lacked range compared to their cross-trial variation and this was reflected in their poor calibration statistics. The four parameters mentioned above have SECVs of similar value to the SD of the reference sample and also less than half the overall population SD. Multiplicative Scattering Correction (MSC) math pre-treatment over a Wave Number range of 7502.1 to 5446.3 cm^{-1} and 4424.1 to 4246.7 cm^{-1} was used for both the ingredient protein and the carbohydrate calibrations with 3 outliers of the 77 samples in the calibration set removed. The calibration for *I-ProFat* used a Min-Max Normalisation math pre-treatment over the ranges 1249.2 to 6098.1 cm^{-1} and 4601.5 to 4246.7 cm^{-1} with 2 outliers removed. For the *I-sAA* calibration a Constant Offset Elimination treatment was used over the range 12493.2 to 6098.1 cm^{-1} and 5450.1 to 4597.7 cm^{-1} with 5 outliers removed.

8.3.2 Ingredient digestibility calibrations

Of the digestibility value calibrations *DV-Pro* and *DV-sAA* both had SECVs commensurate with the standard errors seen in the Myallie reference data (See Table 8.2.) but for *DV-E* the SECV was too high. However the SECV values of all the calibrations were not really low enough relative to the variability across the whole population. The best digestibility calibration was for *DV-Pro* which had a SECV of 2.7% with a mean of 42.4% ($R^2 = 0.472$). This compares to the reference sample standard deviation of 3.6% with a mean of 41.4%. The standard deviation of the trial population for *DV-Pro* was 4.3%, or just less than twice the SECV. For this calibration the math pre-treatment was Straight Line Subtraction with a Wave Number range of 1249.3 to 9295.7 cm^{-1} with 2 of the 77 samples removed as outliers.

8.3.3 Inter-correlation of digestibility, nutrient and energy values

Table 8.3 was compiled from the reference nutrient composition, energy value and digestibility values of the samples used in the NIRS calibrations. That is, some values were removed, as they were not part of the calibration sets. Table 8.4 is the corresponding correlation matrix of the prediction data. Comparison of the tables shows that there is trend for of slightly greater cross-correlation between the NIRS prediction data than between the reference data. For example: on the basis of the reference data there was a correlation of 0.685 between ingredient protein (*I*-Protein) and digestible protein value (*DV-Pro*) but based on the NIR predictions the correlation is 0.792. However, where there are strong inter-correlations in the reference data, (e.g. between *I-Protein* and *I-ProFat*, *I-CHO* and *I-sAA*.) this is also reflected in the prediction data.

8.4 Discussion

8.4.1 General comments

Previous work has shown the suitability of NIRS for predicting protein (and by inference total amino acids and carbohydrates) and oil in *L. angustifolius* seed (Burridge, 2007). The NIRS calibrations developed in this study confirm that these compositional components can be successfully predicted in lupin kernel meal but not all the parameters of interest in the feeding trails could be determined (including dry matter, energy and most compositional digestibility parameters). The calibration

results should however be viewed as preliminary in that the data sets are not ideal for every parameter and all possible math pre-treatments have not been applied to the data. In each case the normal NIRS practice of applying the pre-treatment giving the lowest SECV value was used in the first instance and adopted if the wave number range suggested was likely to be suitable. Also the calibrations have not been validated by an independent sample test set. However, the cross validation tests do provide a valid indication of the potential of the calibrations.

Overall the standard errors of cross validation of the parameters investigated were in most cases commensurate with the cross-trial variation as indicated from the reference sample (standard deviations of 7 samples of cv Myallie). The deficiencies, where they occur, were due to a lack of range and variability in the population of the calibration sets.

8.4.2 Ingredient composition calibrations

Table 8.1 details the calibration and reference statistics and these indicate that viable calibrations were obtained for *I-Protein*, *I-ProFat*, *I-CHO* and *I-sAA*. These four ingredient composition parameters clearly satisfy the requirements for successful prediction (SECVs commensurate with the reference standard error and half or less the cross-trial standard deviation). It is significant that these parameters are all protein-related (strong negative correlation with *I-CHO*). The calibration for *I-Energy* was just short of satisfactory in that it had a similar SECV to the reference error but there was not enough variation and range across the population to be confident of a successful prediction model. However, while short of the essential requirements of a viable NIRS calibration, it did show indications that it may succeed with a calibration set with a slightly greater range of values. The calibration for *I-DM* failed due to its SECV being only marginally less than the standard deviation of the population and having a very narrow range of values (90.9% – 92.8%).

8.4.3 Digestibility calibrations

As expected the digestibility calibration statistics (Table 8.2.) were not as good as those obtained from the original composition data. All the calibrations except *DV-E* had SECVs at about the level of error in the reference results as indicated by the standard deviations of the control. Again the limiting factor was a lack of range and variability in the data sets. Only the protein based parameters *DV-Pro* and *DV-sAA* had a reasonable range relative to the standard deviation, and only the former had a SECV low enough for a possible successful calibration in terms of this variation. Relatively poor R^2 values were also evident for all ingredient digestibility value parameters compared to the original composition and energy data indicating that the NIRS found it difficult to distinguish the values against the background variation. Thus the Digestible Protein calibration appears just short of being viable at this stage but all three have reasonably low SECVs and would be greatly improved by a broader data set.

8.4.4 Data cross-correlations

The calibration models discussed above were used to generate prediction data tables for ingredient and digestibility parameters. These results were cross-correlated using the Excel statistical analysis tool “Correlation”. This measures the relationship between the sets of data. The population correlation calculation is the covariance of two data sets divided by the product of their standard deviations. The original data (excluding data not used in calibration data sets) from the reference methods was treated in the same way. The aim was to check for the occurrence of inter-relationships that were significantly different than those existing in the original data.

As Table 8.3 shows there are strong inter-relationships between some parameters in the reference data. For example *I-CHO* has a significant negative correlation with all the protein based parameters including *I-ProFat* and *I-sAA* and all the protein derived data is strongly cross-correlated. The strongest correlations involving the ingredient digestibility values are between *DV-Pro* and *I-sAA* (0.721) and between *DV-Pro* and *I-ProFat* (0.693). Both these correlations are also present in the NIRS prediction data.

Comparing Table 8.3 and Table 8.4 it is obvious that there is more inter-correlation between the prediction data (particularly involving the ingredient energy value predictions) than in the reference data. The presence of inter-correlations in NIRS calibrations is not unusual given that all the regressions are based on very similar spectral information (although possibly different samples excluded as outliers) and often using the same math treatments and wave number ranges. Even so there is general agreement between the two tables with the protein-based parameters similarly related and most of the digestibility parameters showing a consistency of relationship. One notable exception is the correlation between *I-Energy* and *I-DM* which had s gone from -0.181 to 0.778 but this is probably explained by the very poor nature of the *I-DM* calibration as discussed in 8.4.3.

8.4.5 Conclusion

This study demonstrates that there is great potential to use NIRS to predict the composition and energy of kernel meal samples of narrow-leaf lupins by scanning the kernel meal before diet preparation. The results also show that it there is potential to predict the likely digestibility of some of these compositional components. In order to improve the composition and energy calibrations further, and make the digestibility calibrations viable, data sets with a broader range for each parameter need to be obtained. There also needs to be sufficient samples to enable suitable subsets (test sets) for each calibration to be available to thoroughly evaluate the robustness of the models.

8.5 References

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Tables and Figures

Table 8.1 Ingredient Composition Data Summary.

	I-DM	*I-Protein*	*I-ProFat*	*I-CHO*	*I-Energy*	*I-sAA*
cv Myallie Mean	91.29	42.98	50.74	45.92	20.63	41.37
cv Myallie SD	0.59	2.54	2.35	2.38	0.16	0.97
Population Range	2.4	17.3	17.1	17.8	1.3	20.5
Population SD	0.57	3.39	3.04	3.27	0.32	3.17
SECV	0.46	1.18	1.35	1.39	0.2	1.47
R^2	0.256	0.858	0.765	0.784	0.551	0.733

Table 8.2 Ingredient Digestibility Value Data Summary.

	DV-Pro	*DV-E*	*DV-sAA*
cv Myallie Mean	41.35	11.5	36.2
cv Myallie SD	3.64	0.52	4.64
Population Range	20.4	4.41	22.8
Population SD	4.3	1	5.59
SECV	2.7	0.75	4.27
R^2	0.472	0.355	0.212

Table 8.3 Nutrient composition, energy value and digestibility cross-correlations.

	I-DM	*I-Protein*	*I-ProFat*	*I-CHO*	*I-Energy*	*I-sAA*	*DV-Pro*	*DV-E*	*DV-sAA*
I-DM	1								
I-Protein	-0.205	1							
I-ProFat	-0.176	0.963	1						
I-CHO	0.215	-0.976	-0.994	1					
I-Energy	-0.181	-0.016	0.174	-0.116	1				
I-sAA	-0.116	0.818	0.825	-0.825	0.204	1			
DV-Pro	-0.068	0.685	0.693	-0.679	0.135	0.721	1		
DV-E	-0.03	0.331	0.368	-0.383	0.221	0.395	0.546	1	
DV-sAA	-0.455	0.592	0.585	-0.591	0.237	0.542	0.499	0.27	1

Table 8.4 Nutrient composition, energy value and digestibility cross-correlations.

	I-DM	*I-Protein*	*I-ProFat*	*I-CHO*	*I-Energy*	*I-sAA*	*DV-Pro*	*DV-E*	*DV-sAA*
I-DM	1								
I-Protein	-0.362	1							
I-ProFat	-0.221	0.931	1						
I-CHO	0.254	-0.978	-0.956	1					
I-Energy	0.778	-0.408	-0.189	0.27	1				
I-sAA	-0.159	0.816	0.871	-0.846	-0.134	1			
DV-Pro	-0.275	0.792	0.842	-0.821	-0.13	0.821	1		
DV-E	-0.169	0.444	0.489	-0.497	-0.034	0.565	0.797	1	
DV-sAA	-0.186	0.708	0.7	-0.683	-0.333	0.733	0.528	0.42	1

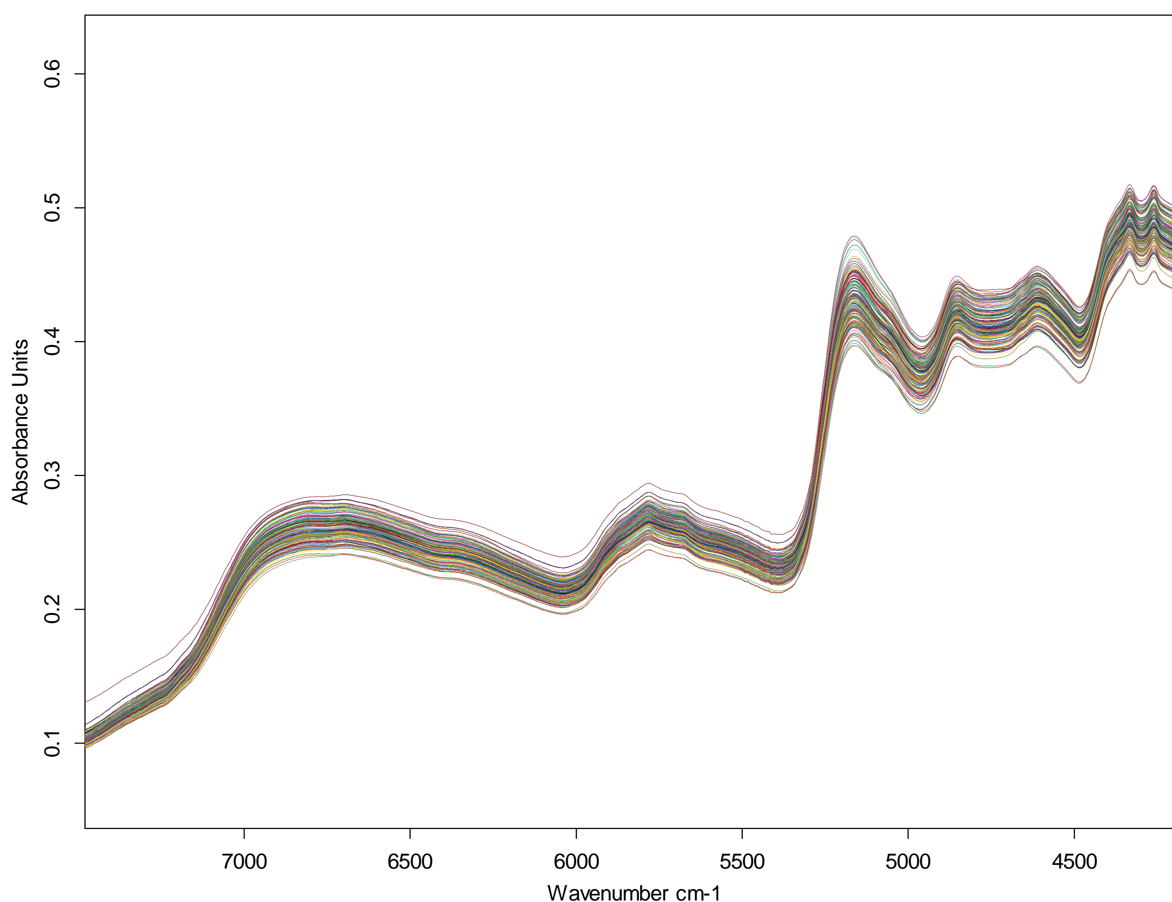

Figure 8.1 Plot of the spectra obtained from the 74 lupin kernel meal samples in the range of approximately 6500 to 4000 cm^{-1}.

9.0 The influence of the dietary inclusion of the alkaloid gramine, on rainbow trout (*Oncorhynchus mykiss*) growth, feed utilisation and gastrointestinal histology[a]

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Abstract

This study examined the influence of the alkaloid gramine, when included in diets for rainbow trout, *Oncorhynchus mykiss*. Quinolizidine alkaloids have been suggested as a potential anti-nutritional problem with the use of lupin (*Lupinus* sp.) meals in aquaculture diets. The findings from the present study show that above a critical threshold, the alkaloid gramine does have a strong anti-palatability effect. The effect is noted at a minimum gramine concentration of 500 mg/kg of diet, though not at 100 mg/kg. A continuing strong anti-palatability response is noted at higher inclusion levels and at the highest gramine inclusion concentration examined in this study (10,000 mg/kg), insufficient feed was consumed to even supply maintenance protein and energy demands. No adaptation to concentrations of gramine was observed throughout the 6-week study. No effects on nitrogen, energy or phosphorus digestibility were seen at the 500 mg/kg inclusion concentration of gramine relative to the reference diet, although the inclusion of the yellow lupin kernel meals (both Wodjil and Teo varieties) in the diet did improve the digestibility of phosphorus. Growth, as assessed using a range of parameters including weight gain, growth rate, nutrient and energy retention of fish fed the experiment treatments was largely consistent with feed intake. Survival of fish was significantly reduced at gramine inclusion levels above 1,000 mg/kg. Food conversion ratio (FCR) and food conversion efficiency (FCE) were also reflective of feed intake and growth levels observed of each treatment. The concentrations of the plasma thyroid hormones tri-iodothyronine (T_3) and thyroxine (T_4) of fish from each of the treatments were consistent with feed intake (including the controls) suggesting that the concentrations of these hormones are in response to feed intake, not specifically the gramine levels in the diets. However, the inclusion of the *L. luteus* kernel meals resulted in a significant change in T_4 levels, with a degree of independence of the feed intake, suggesting that there may be another mechanism by which these meals are influencing the concentrations of this hormone. In this study there was an increase in the density of melano-macrophage centres (MMC) with high dietary levels of gramine. However, in the absence of any histological evidence for a toxic effect, it is likely that the increased MMC densities observed in the fish fed high concentrations of gramine are associated with starvation. This study demonstrated that the lupin alkaloid gramine, can have a strong anti-nutritional effect on fish at inclusion concentrations greater

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than 100 mg/kg, but that its mode of action is primarily through an anti-palatability effect. It is therefore considered unlikely that alkaloid effects would be observed in diets even with 50% inclusion of kernel meals from Australian commercial *L. luteus* varieties.

9.1 Introduction

It is well recognised in the aquaculture feeds industry that there is a need to reduce reliance on fish meal in aquaculture feeds (Naylor et al., 1999). Increasing the actual or prospective utilisation of other protein meals in diets for aquatic species, substantial risk reduction is achieved. The use of plant protein meals as alternative protein resources has been well studied and many viable options including soybean, glutens and lupin meals have been adopted industrially (Carter and Hauler, 2000; Storebakken et al., 2000; Glencross et al., 2004). However, the introduction of anti-nutritional factors and other biologically active compounds can accompany the use of plant protein meals (Francis et al., 2001).

Anti-nutritional factors (ANF) can affect the utilisation of food by an animal through several avenues, including the metabolic axis, nutrient digestibility or ingredient palatability (Refstie et al., 1998, 1999; Glencross et al., 2003a, b). Alkaloids are heterocyclic amino acid derivatives produced by plants as a chemical defence mechanism. While alkaloids are found in most legume species, they have traditionally been found in high concentrations in the seeds of plants from the *Lupinus* genus (Petterson et al., 1997; Wasileswko and Buraczewska, 1999). Notably, a variety of alkaloids are found in these seeds. In some varieties of the species *Lupinus luteus* a major alkaloid component is gramine (Petterson, 2000). Feeding studies with kernel meals from the seeds of *L. luteus* have shown good prospect for their use in aquaculture feeds because of their high digestible protein content, although some deterioration in growth performance at high inclusion levels has been noted (Glencross et al., 2004).

Consumption of gramine at toxic levels in mice has been noted to lead to psychotropic levels of excitement and seizure. The mode of action for gramine as an ANF, or toxicity data on this compound is limited. However mammalian effects include changes in tubules and glomeruli in the kidney, ureter and bladder, endocrine changes in spleen weight, and biochemical changes such as enzyme inhibition, induction via changes in blood or tissue levels of phosphatases (TXCYAC, 1980), although no specific data is available for any fish species. Tolerance concentrations to the inclusion of dietary gramine in other vertebrate species (rats, pigs and poultry) have been determined at; about 300 mg/kg for rats, > 500mg/kg diet for pigs and about 650 mg/kg diet for poultry (Pastuszewska et al., 2001). The effects of concentrations as low as 250 mg/kg of *L. angustifolius* alkaloids have been reported in rats (Butler et al., 1996), although concentrations of alkaloids from *L. albus* were only reported to have an adverse effect at 320 mg/kg (Zdunczyk et al., 1998).

The current Australian commercial *L. luteus* variety (Wodjil) has very low gramine concentration compared to European varieties such as Teo. However, Wodjil has proven agronomically costly to produce because of the high levels of insecticide use required to deal with substantial insect infestation problems (Perry et al., 1998; Berlandiet and Sweetingham, 2003). There is evidence that aphid infestation is directly related to the low inherent concentration of gramine (Risdall-Smith et al., 2004). Higher alkaloid varieties of *L. luteus*, such as Teo, have better resistance to insect infestation, but it is unclear whether the higher alkaloids will influence the usefulness of the kernel meal as an aquaculture feed ingredient.

This study reports on the nutritional influence of gramine on the feed intake, growth, some

biochemical parameters and tissue histology of rainbow trout, *Oncorhynchus mykiss.* This was examined over a range of inclusion concentrations above and below naturally occurring concentrations found in domesticated varieties of *L. luteus*.

9.2 Methods

9.2.1 Ingredients and diet preparation

Purified gramine was purchased (Aldrich catalogue No 1080 – 6, 99% purity). The gramine was dissolved in methanol and was added to a methanol saturated cellulose slurry and the mixture was thoroughly mixed. The solvent was removed *in vaccuo* and the gramine/cellulose mixture was dried under vacuum. Cellulose was used as a carrier for the gramine allowing for easy dispersion of the gramine in the individual diets. The gramine/cellulose mixture was added to the experimental diets according to the formulations in Table 9.1. All ingredients were ground such that they passed through a 600 µm screen. All experiment diets were formulated to be isonitrogenous (400 g/kg) and isoenergetic (19.5 MJ/kg) on a digestible nutrient basis. Digestibility coefficient values for key ingredients were based on those reported earlier (Glencross et al., 2005). Diets were processed by the addition of water (about 30% of mash dry weight) to all ingredients while mixing to form a dough. This dough was subsequently screw-pressed through a 3 mm diameter die using a pasta maker. The resultant moist pellets were then oven dried at 70°C for approximately 24 h before being air-cooled, bagged and stored at -20°C. The feed intake deterrent, sulfamerazine sodium was added to two diets, based on the reference diet, at different levels to create a series of negative controls (Boujard and Le Gouvello, 1997). Ingredient composition, diet formulations and diet composition are presented in Tables 9.1, 9.2 and 9.3 in that respective order.

9.2.2 Chemical analysis

All chemical analyses were contracted out to professional chemical analytical laboratories. Respective samples of diet, faecal and whole-body samples were analysed for a variety of analytes, depending on experiment, including dry matter, chromium, ash, fat, nitrogen, phosphorus and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Chromium and phosphorus levels were determined using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Kjeldhal digestion, based on N x 6.25. Crude fat content was determined gravimetrically following extraction of the lipids according to the crude fat procedure (AOAC, 1990). Ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Organic matter content was determined based on the difference between dry matter content minus ash content. Gross energy was determined by adiabatic bomb calorimetry. Concentrations of tri-iodothyronine (T_3) and thyroxine (T_4) were determined by a competitive immunoassay method using chemiluminescence detection (Fisher, 1996). Gramine concentrations were determined by extraction with trichloroacetic acid and then extracted from the aqueous layer with methylene chloride. The gramine concentration was measured by gas chromatography using a capillary column (HP1, 30 metres) and detected by a flame ionisation detector (Harris and Wilson, 1988).

9.2.3 Fish management

Forty-eight shallow-conical bottomed 250 L tanks, with flow-through freshwater (4 L/min, salinity < 1 PSU and 14.1 ± 0.8°C, dissolved oxygen 9.7 ± 0.3 mg/L; mean ± SD, n=42), were each stocked with 24, individually weighed, juvenile (9 month, 51.7 ± 0.58 g; mean ± SD) hatchery reared rainbow trout (Pemberton Strain; Molony et al., 2004). Treatments were randomly assigned in quadruplicate to the tank array. Photoperiod was maintained at 12L: 12D.

The fish were fed to apparent satiety once daily at about 0800 h for 42 days. Apparent satiety, as determined by a loss in feeding activity, was reached after three feeding sessions over a 1 h period. Uneaten feed was removed from each tank 1 h later and the uneaten portion dried and weighed to allow the determination of daily feed intake based on correction factors for leaching losses sustained over an equivalent period.

Fish were individually re-weighed after three and six weeks, with all fish within each tank used to determine the average weight gain per tank and treatment. Five fish were taken as an initial sample for composition analysis. At the end of the study three fish were taken from each tank (4 replicates x 3 fish, per treatment) for whole body analysis. An additional three fish from each tank were sampled for blood biochemistry, within 1 min of capture, by caudal tail vein puncture using a 1 ml syringe fitted with at 20G needle. Growth was assessed as mean weight gain and daily growth coefficient (DGC). DGC was calculated as (Kaushik, 1998):

$$DGC = \frac{\left(W_f^{1/3} - W_i^{1/3}\right)}{t} \times 100$$

9.2.4 Digestibility assessment

At the end of the trial faeces were collected using stripping techniques based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing AQI-S™ (AQI-S NZ Ltd, Lower Hutt, New Zealand) (0.02 mL/L) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not contaminated by urine and mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial on ice and later stored in a freezer at -20°C. Faeces were freeze dried prior to analysis. Sufficient faecal sample for analysis could not be obtained from some treatments, primarily because of low feed intake in some treatments.

Differences in the ratios of the parameters of protein or gross energy to chromium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1969):

$$ADC_{diet} = 1 - \left(\frac{Cr_{diet} \times Parameter_{faeces}}{Cr_{faeces} \times Parameter_{diet}} \right)$$

where Cr_{diet} and Cr_{faeces} represent the chromium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (protein or energy) content of the diet and faeces respectively.

9.2.5 Tissue histology

Two fish from each tank (n = 2 x 4 per treatment) were euthanised with a sharp cranial blow at week three of the study and fixed in 10% neutral buffered formalin. Incisions were made in the fish's abdominal wall to allow penetration of the formalin. Following preservation the fish were dissected and samples of their liver, kidney, spleen, pyloric caeca and intestine were taken for histological examination. The samples were embedded in paraffin, sectioned at 5 µm and stained with haematoxylin and eosin using standard techniques. A representative kidney section was stained with Perls stain for iron, Ziehl-Neelson for lipofuscin and Masson Fontana for melanin, using standard techniques.

The sample sections were examined for lesions. A digital image (Olympus DP11) at 200x magnification was taken of each kidney sample and the density of melano-macrophage centres and pigment deposits in the spleen were scored for each of the prints (1 = few to 4 = abundant). Scoring was performed without access to the nutrition data, and repeated by three independent readers.

9.2.6 Statistical analysis

All figures are mean ± SE unless otherwise specified. Data were analysed for homogeneity of variances using Cochrans test. Effects of diets were examined by ANOVA using the software package Statistica (Statsoft®, Tulsa, OK, USA). Levels of significance were determined using Tukeys HSD test, with critical limits being set at $P < 0.05$. Effects of inclusion level of gramine on key performance parameters were examined by linear and non-linear regression modeling, also using the software package Statistica. Variation between scorers for tissue histology was examined using Friedman two-way ANOVA (Systat®, Richmond, CA, USA) and variation between trials was compared using Kruskal-Wallis analysis of variance (Systat®).

9.3 Results

9.3.1 Influence of gramine on feed intake

One of the primary features noted with the increasing inclusion of gramine in the diet of the rainbow trout was the deterioration of feed intake with levels above 100 mg/kg DM (Table 9.4). The negative controls (C1 and C2) also had significantly poorer feed intake over the course of the experiment than the Reference diet (no gramine, no sulferamerazine, no lupin diet) and several of the lower level gramine inclusion diets. Feed intake by fish fed the Wodjil diet was equivalent to that of fish fed the reference diet. Feed intake by fish fed the Teo diet was significantly less than that of fish fed the reference diet. Feed intake by fish fed diets that had a blend of Wodjil and Teo also had significantly poorer feed intake, but not as low as that observed with Teo alone.

Palatability responses to the gramine diets were rapid and observed within a matter of days (Figure 9.1). No adaptation to the gramine levels was observed during the course of the experiment as was noted by the relative feed intakes during the first and sixth weeks of the experiment (Table 9.4).

9.3.2 Influence of gramine on feed digestibility

Digestibility assessment of complete diets showed that at low inclusion levels (< 500 mg/

kg), that gramine did not influence the digestibility of nitrogen, energy or phosphorus (Table 9.5). Because of poor diet palatability sufficient faecal samples could not be obtained from the treatments with gramine levels higher than 500 mg/kg.

Inclusion of the yellow lupin kernel meals (both Wodjil and Teo varieties) into the diet did not significantly affect either the nitrogen or energy digestibility, but significantly increased the digestibility of phosphorus in the diets compared to the reference diet (Table 9.5).

9.3.3 Influence of gramine on fish growth and feed utilisation

Growth of fish fed the experiment treatments was largely consistent with feed intake. No effect on growth by the inclusion of gramine levels below 500 mg/kg levels was observed. From 500 mg/kg and above a dramatic decline in growth was noted (Table 9.4). This effect on growth was consistent for both weight gain and DGC. A similar decline in growth was noted with both of the negative controls (C1 and C2) (Table 9.4). Growth of fish fed the Wodjil diet was not significantly different from that of the reference diet (Table 9.4). However, the inclusion of Teo kernel meal significantly reduced growth. A blend of Teo and Wodjil resulted in growth mid-way between that observed for the two discrete varieties (Table 9.4).

Survival of fish was significantly reduced at gramine inclusion levels above 1,000 mg/kg. Poorer survival was also noted from the Teo treatment (Table 9.4). No other significant differences among treatments were noted.

Feed conversion ratio (FCR) and feed conversion efficiency (FCE) were reflective of feed intake and growth levels observed of each treatment. No significant differences between the reference diet and all treatments up to and including 500 mg/kg were noted (Table 9.4). The FCR continued to increase with increasing gramine level up to 1,500 mg/kg. The 10,000 mg/kg treatment had negative growth and accordingly the fish had a negative FCR (Table 9.4).

The FCR of fish fed the Wodjil diet was not significantly different from that of the reference diet (Table 9.4). However, the inclusion of Teo kernel meal resulted in a significantly poorer FCR and FCE. A blend of Teo and Wodjil resulted in FCR/FCE mid-way between that observed for the two discrete varieties (Table 9.4).

Nitrogen and energy retention by fish fed the treatments was also largely consistent with feed intake. No effect on nitrogen retention by the inclusion of gramine below 1,000 mg/kg levels was observed, however at 500 mg/kg a deterioration in the energy retention was noted relative to that of the reference diet. From 1,000 mg/kg and above, deterioration in both nitrogen and energy retention was noted (Table 9.4). A similar decline in energy retention was noted with both of the negative controls (C1 and C2) (Table 9.4). Nitrogen and energy retention by fish fed the Wodjil diet was not significantly different from that of the reference diet (Table 9.4). However, the inclusion of Teo kernel meal significantly reduced retention efficiency of both nitrogen and energy. A blend of Teo and Wodjil resulted in a significant reduction in energy retention, but did not affect nitrogen retention (Table 9.4).

The concentrations (pmol/l) of the thyroid hormones tri-iodothyronine (T_3) and thyroxine (T_4) of fish fed the treatments was also largely consistent with feed intake (g/tank) (y_{T3}= 0.0565x + 3.6343, R^2 = 0.8441 and y_{T4}= 0.0368x + 1.6523, R^2 = 0.7634). No effect on either T_3 or T_4 concentrations was noted with the inclusion of gramine below 500 mg/kg levels. From 500 mg/kg and above, deterioration in T_4 concentrations were noted and above 1000 mg/kg a deterioration in T_3 concentrations was noted (Table 8.4). A similar decline in T_3 and

T_4 concentrations was noted with the higher inclusion concentration of sulfamerazine sodium in the negative controls (C2), but not at the lower inclusion concentration (C1) (Table 9.4). T_4 concentrations from fish fed the Wodjil diet were significantly less than those from fish fed the reference diet, but no effects on T_3 were noted (Table 9.4). However, the inclusion of Teo kernel meal significantly reduced both T_3 and T_4 concentrations. A blend of Teo and Wodjil resulted in a significant reduction in T_4 concentrations, but did not affect T_3 concentrations (Table 9.4).

9.3.4 Influence of gramine on histology

The dark brown-black deposits did not stain for iron or lipofuscin but did stain strongly for melanin. No lesions considered to represent significant changes in health status were detected in the liver, kidney, spleen, pyloric caeca or intestine. Melano-macrophage centres (MMC) are normally found in the kidney and are characterised as dark brown-black macrophage aggregations of variable size and shape, however, large variations were observed in the density of MMC in the haematopoetic tissue in the kidney samples. These were scored independently and between reader scores were tested using Friedman two-way ANOVA. There was no evidence of systematic variation between readers ($P < 0.001$, 2df, Friedman test statistic = 21.458). Variation in scores between treatments was significant for each reader (Reader 1, $P < 0.001$, 11 df, Kruskal Wallis test statistic=31.155; Reader 2, $P < 0.0001$, 11 df, Kruskal Wallis test statistic=38.826; Reader 3, $P = 0.056$, 11 df, Kruskal Wallis test statistic=19.302) (Table 9.6). The difference is driven by treatments 6 and 7, where all readers awarded consistently high scores.

9.4 Discussion

Any compound feed for an animal is generally only as valuable as the sum of the value of its ingredients. The key value in an ingredient such as lupin kernel meal is its protein and/or energy content. However, for most animals the use of plant protein resources often introduces problems associated with the inherent anti-nutritional content of these resources. Alkaloids have been touted as a potential anti-nutritional problem with the use of lupin meals in aquaculture diets, despite the previous lack of reliable data to confirm or refute this reputation (Francis et al., 2001).

9.4.1 Influence of gramine on feed intake

Alkaloids are generally believed to exert their anti-nutritional effect through inhibition of palatability at the lower inclusion concentrations, although other bioactive effects have been suggested at higher inclusion concentrations. The findings from the present study confirm that above a critical threshold, the alkaloid gramine does have a strong anti-palatability effect. The effect is noted at a minimum gramine concentration of 500 mg/kg of diet, though not at 100 mg/kg. A continuing strong anti-palatability response is noted at higher inclusion concentrations and at the maximum gramine inclusion concentration examined in this study (10,000 mg/kg) insufficient feed was consumed to even supply maintenance protein and energy demands. This compares well with other species like rats, pigs and poultry (Pastuszewska et al., 2001), but shows that fish are slightly more sensitive in their palatability of gramine than either pigs or poultry at least, and possibly rats too.

In undomesticated varieties of other lupin species, such as *L. angustifolius* and *L. cosentii*, total alkaloid concentrations exceeding 30,000 mg/kg have been reported (Petterson, 2000). However, in

Australia, modern domesticated varieties of *L. angustifolius* are not made available for commercial release if total alkaloid concentrations exceed 200 mg/kg (Gladstones, 1998; Perry et al., 1998). This has largely negated alkaloid related problems being observed in animal feed industries, at least from Australian grown lupins. It should be noted that the *L. angustifolius* (angustifoline, lupanine, α-isolupanine and 13- hydroxy lupanine) and *L. cosentii* (epilupinine, epilupine-N-oxide and multiflorine) species of lupin have a totally different alkaloid profile to *L. luteus*. However, no fish feeding trials have been carried out using the alkaloids in *L. angustifolius* species.

9.4.2 Influence of gramine on feed digestibility

The observation that no effects on nitrogen, energy or phosphorus digestibility were seen at the 500 mg gramine/kg diet inclusion concentration, relative to the reference diet suggests that the alkaloid effect is not inhibiting the animal's ability to absorb nutrients and energy from the diet once it is ingested. Although not specifically related to the alkaloid effect, the inclusion of the yellow lupin kernel meals (both Wodjil and Teo varieties) into the diet did improve the digestibility of phosphorus in the diets compared to the reference diet and this has been noted in other studies on the digestibility assessment of lupin kernel meals (Glencross and Hawkins, 2004; Glencross et al., 2005).

9.4.3 Influence of gramine on fish growth

Growth, as assessed using a range of parameters including weight gain, growth, nutrient and energy retention, of fish fed the experiment treatments was largely consistent with feed intake. Survival of fish was also significantly reduced at gramine inclusion levels above 1,000 mg/kg and was believed to result from an inability of the fish to survive the experimental period with such a low level of feed intake. Feed conversion ratio (FCR) and feed conversion efficiency (FCE) were also reflective of feed intake and growth levels observed of each treatment.

That the levels of the plasma thyroid hormones tri-iodothyronine (T_3) and thyroxine (T_4) of fish fed the treatments were also largely consistent with feed intake across all experiment treatments suggests that the levels of these hormones are in response feed intake, not specifically the gramine levels. However, the observation that the inclusion of the *L. luteus* kernel meals resulted in a significant change in T_4 levels, with a certain degree of independence of the feed intake levels, suggests that there may be another mechanism by which these meals are influencing the levels of this hormone. This contrasts results from earlier work examining the use of *L. luteus* kernel meal, where no significant alterations to the thyroid hormones were noted (Glencross et al., 2004). However, in contrast to that study the present study used plasma rather than whole blood samples and this may have had significant effects on the reliability of the assays being used. The findings are also consistent with work by Burel et al. (1998), who observed changes in thyroid hormone levels with the inclusion of *L. albus* kernel meal. Another study by Gomez et al. (1997), using commercial pellets showed no relationship between plasma thyroid hormones and feed intake (%BW), though did show positive a relationship against growth rate (SGR) in rainbow trout, similar to that observed in the present study.

9.4.4 Influence of gramine on histology

Melano-macrophage centres are normally found in the liver and kidney of trout where they are involved in trapping and removal of cellular debris and cellular toxicants as well as storage of effete materials and recovery of iron (Agius, 1985; Agius and Roberts, 2003). In this trial there was an increase in the density of MMC with high dietary levels of gramine. MMC

increase in incidence with age, however, starvation, exposure to environmental contaminants and pathological conditions resulting in cellular damage also increase the incidence of MMC (Agius and Roberts, 1981; Wolke, 1992; Capps et al., 2004). The MMC in this trial were not associated with haemosiderin or lipofuscin but did stain strongly for melanin. Nevertheless, the density of MMC aggregations is a useful bioindicator of fish health (Blazer et al., 1987; Capps et al., 2004). In the absence of any histological evidence for a toxic effect, it is likely that the increased MMC densities observed in the fish fed high levels of gramine are associated with starvation.

9.4.5 Conclusion

This study demonstrates that the lupin alkaloid gramine, can have a strong anti-nutritional effect on fish, at certain critical inclusion levels. Although these inclusion levels exceed 100 mg/kg and are unlikely to be observed in diets even with 50% inclusion of kernel meals from Australian commercial varieties of either *L. luteus* or *L. angustifolius*. It is hypothesised that the primary mode of action of gramine is through an anti-palatability effect that has secondary consequences for growth, nutrient utilisation, metabolic hormones and kidney histology.

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Tables and Figures

Table 9.1 Composition of the ingredients (all values are g/kg DM unless otherwise stated).

Nutrient	[a]Fish meal	[b]Pregelled wheat starch	[c]Cellulose	[d]Wodjil	[e]Teo
Dry matter content (g/kg)	917	906	933	924	920
Crude protein	770	7	3	512	541
Crude fat	68	11	2	79	79
Ash	142	3	2	54	73
Crude fibre	0	10	660	33	35
Phosphorus	22	0	0	6	7
Organic matter	858	997	998	946	927
Gross energy (MJ/kg DM)	21.3	17.2	17.3	20.9	20.9
Alkaloids (mg/kg DM)	0	0	0	32	4087
Arginine	43	0	0	47	61
Histidine	25	0	0	14	14
Isoleucine	28	2	0	17	20
Leucine	55	0	0	35	43
Lysine	46	1	0	23	17
Methionine	21	0	0	4	3
Phenylalanine	29	0	0	18	21
Threonine	32	2	0	16	19
Valine	34	0	0	17	19

[a] Chilean Anchovy meal supplied by Skretting Australia, Cambridge, Tasmania, Australia.

[b] Supplied by Weston BioProducts, Henderson, Western Australia, Australia.

[c] Supplied by ICN Biomedical, Costa Mesa, CA, USA.

[d] Supplied by Coorow Seed Cleaners Pty Ltd, Coorow, Western Australia, Australia. [e] Supplied by Department of Agriculture, South Perth, Western Australia, Australia.

Table 9.2 Formulations of the diets (all values are g/kg).

Ingredient	0	10	100	500	1,000	1,500	10,000	Neg - 1	Neg - 2	Wodjil	Teo	Blend
Chromium oxide	5	5	5	5	5	5	5	5	5	5	5	5
Pre-mix vitamins and minerals*	5	5	5	5	5	5	5	5	5	5	5	5
Cellulose	113	112.9	112	108	103	98	13	108	103	22	30	26
Cellulose + 100,000 mg/kg alkaloids	0	0.1	1	5	10	15	100	0	0	0	0	0
Pregelled starch	90	90	90	90	90	90	90	90	90	90	90	90
Fish oil	166	166	166	166	166	166	166	166	166	170	171	170.5
Fish meal	621	621	621	621	621	621	621	621	621	408	399	403.5
L. luteus cv. Wodjil kernel meal	0	0	0	0	0	0	0	0	0	300	0	150
L. luteus cv. Teo kernel meal	0	0	0	0	0	0	0	0	0	0	300	150
Sulfamerazine sodium	0	0	0	0	0	0	0	5	10	0	0	0

Source of ingredients provided in table 1.

* Supplied by Rhone Poulenc, Goodna, Queensland, Australia. Vitamin and mineral premix includes (IU/kg or g/kg of premix): Retinol, 2.5MIU; Cholecalciferol, 0.25 MIU; α-tocopherol, 16.7 g; Menadione, 1.7 g; Thiamine, 2.5 g; Riboflavin, 4.2 g; Niacin, 25 g; Pantothenic acid, 8.3; Pyridoxine, 2.0 g; Folic acid, 0.8; Methylcobalamine, 0.005 g; Biotin, 0.17 g; Ascorbic acid, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g;

Table 9.3 Composition of the diets (all values are g/kg DM unless otherwise stated).

Ingredient	0	10	100	500	1,000	1,500	10,000	C1	C2	Wodjil	Teo	Blend
Dry matter	960	954	945	951	949	959	966	950	951	958	950	957
Protein	419	426	418	424	416	429	417	426	426	448	436	447
Fat	231	231	232	228	227	228	230	228	227	203	217	209
Phosphorus	17	17	17	16	17	16	16	17	17	15	13	14
Ash	104	104	104	108	111	114	120	115	108	118	118	108
Gross Energy (MJ/kg DM)	22.88	23.44	23.17	23.25	23.12	23.35	23.52	23.24	23.41	23.16	23.48	23.53
Alkaloids (mg/kg)	0	10	116	547	1117	1867	11594	0	0	11	1179	512

Table 9.4 Growth, feed intake, survival, utilisation efficiencies and hormonal levels of fish fed the experiment diets.

	0	10	100	500	1,000	1,500	10,000	C1	C2	Wodjil	Teo	Blend	Pooled SEM
Initial weight (g/fish)	51.7 [a]	51.9 [a]	51.5 [a]	51.6 [a]	51.3 [a]	52.0 [a]	51.7 [a]	52.0 [a]	51.6 [a]	51.6 [a]	52.0 [a]	51.3 [a]	0.08
Final weight (g/fish)	172.7 [a]	179.4 [a]	178.8 [a]	108.1 [c]	59.9 [d]	51.7 [de]	45.3 [e]	143.8 [b]	119.7 [c]	186.9 [a]	61.8 [d]	108.2 [a]	7.61
DGC (%/d)	4.39 [a]	4.55 [a]	4.49 [a]	2.47 [c]	0.46 [d]	-0.02 [de]	-0.38 [e]	3.58 [b]	2.87 [c]	4.74 [a]	0.52 [d]	2.49 [c]	0.27
Gain (g/fish)	121.1 [a]	127.5 [a]	127.3 [a]	56.6 [c]	8.6 [d]	-0.3 [de]	-6.4 [e]	91.8 [b]	68.1 [c]	135.2 [a]	9.8 [d]	56.9 [c]	7.61
FCR (g feed: g gain)	0.83 [a]	0.79 [a]	0.80 [a]	0.77 [a]	2.61 [b]	3.56 [c]	-1.04 [d]	0.91 [a]	0.96 [a]	0.78 [a]	2.75 [b]	0.72 [a]	0.45
FCE (g gain: g feed)	1.21 [ab]	1.26 [a]	1.24 [a]	1.30 [a]	0.38 [c]	0.28 [c]	-0.96 [d]	1.10 [ab]	1.05 [b]	1.28 [a]	0.36 [c]	1.40 [a]	0.10
Food intake (g/fish/d) – week 1	1.74 [a]	1.68 [a]	1.73 [a]	1.14 [a]	0.51 [c]	0.49 [cd]	0.32 [d]	1.57 [ab]	1.22 [b]	1.76 [a]	0.68 [c]	1.09 [b]	0.09
Food intake (g/fish/d) – week 6	3.12 [a]	3.28 [a]	3.27 [a]	1.19 [d]	0.28 [e]	0.15 [e]	0.09 [e]	2.65 [b]	1.96 [c]	3.40 [a]	0.23 [e]	1.02 [d]	0.20
Food intake (g/fish) – Total	100.1 [a]	101.1 [a]	102.4 [a]	43.1 [d]	12.9 [e]	11.4 [e]	6.7 [e]	83.4 [b]	65.0 [c]	105.6 [a]	16.6 [e]	40.6 [d]	5.61
Survival (%)	100.0 [a]	100.0 [a]	98.6 [a]	98.6 [a]	94.4 [b]	79.2 [d]	86.1 [c]	98.6 [a]	98.6 [a]	98.6 [a]	83.3 [c]	97.2 [ab]	1.29
Nitrogen retention (%)	42.2 [a]	42.1 [a]	44.4 [a]	44.6 [a]	21.6 [b]	0.4 [c]	-30.5 [d]	40.7 [a]	36.6 [a]	44.3 [a]	17.6 [b]	44.1 [a]	3.39
Energy retention (%)	60.1 [a]	54.4 [a]	54.6 [a]	45.5 [b]	11.0 [d]	2.9 [e]	-32.0 [f]	44.2 [b]	39.6[b]	58.8 [a]	8.2 [de]	27.6 [c]	4.43
Plasma free T_3 (pmol/l)	9.6 [a]	8.1 [b]	10.6 [a]	7.1 [bc]	4.0 [d]	3.5 [de]	3.1 [e]	9.0 [ab]	6.7 [c]	8.3 [b]	5.1 [cd]	7.4 [bc]	0.44
Plasma free T_4 (pmol/l)	5.8 [a]	5.6 [a]	5.9 [a]	3.1 [b]	2.4 [bc]	2.0 [c]	1.4 [d]	6.2	3.5 [b]	3.6 [b]	2.3 [bc]	3.6 [b]	0.33

Table 9.5 Digestibility (%) of protein, energy and phosphorus from experimental diets.

Treatment	ADC-Protein	ADC-Energy	ADC-Phosphorus
0	87.2 [a]	84.0 [a]	29.0 [a]
100	86.7 [a]	86.0 [a]	27.8 [a]
500	88.0 [a]	86.8 [a]	31.6 [a]
Wodjil	88.6 [a]	87.3 [a]	54.7 [b]
Blend	87.2 [a]	84.0 [a]	51.6 [b]
Pooled SEM	0.26	0.56	2.91

Table 9.6 Combined counts of scores (columns, 1=few, 4= abundant) awarded by three independent readers to the number of melano-macrophage centres in kidneys of fish in different treatments (rows, 1-12). Kidneys of eight fish were examined for each treatment except for treatment 10 (=7 fish).

Treatment	Scores			
	1	2	3	4
1	13	10	1	0
2	5	14	5	0
3	8	9	6	1
4	7	12	4	1
5	4	7	8	5
6	0	4	15	5
7	0	7	9	8
8	8	11	5	0
9	10	11	2	1
10	8	10	3	0
11	5	8	10	1
12	8	7	8	1

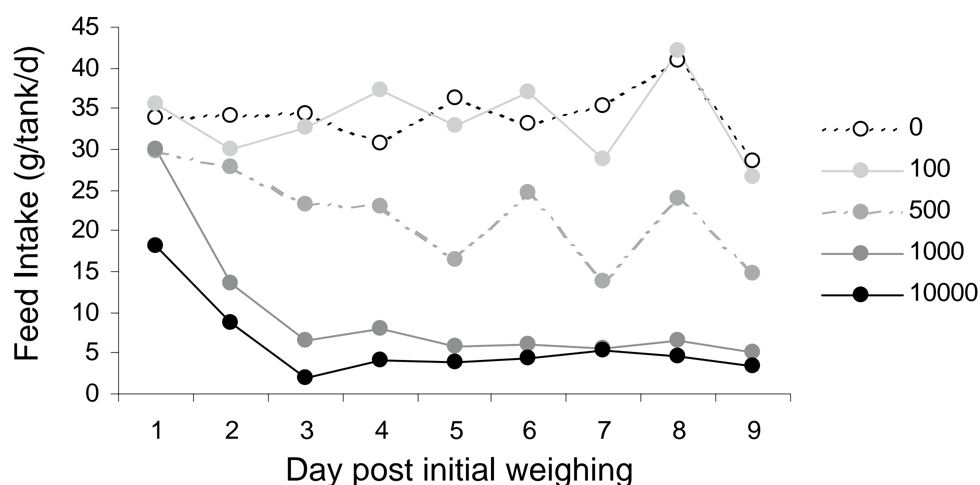

Figure 9.1 Daily mean feed intake by tank, of each treatment, over the first nine days of the experiment. Poorest feed intake was observed with the 10,000 mg/kg treatment, which not significantly different from that of the 1,500 mg/kg treatment. The 500 mg/kg treatment was significantly better than both the 1,500 and 10,000 mg/kg treatments, but significantly poorer than the 100 mg/kg diet and the reference (0 mg/kg) treatments. No significant differences were noted between the reference and 100 mg/kg treatments.

10.0 Evaluation of the influence of *Lupinus angustifolius* kernel meal on dietary nutrient and energy utilisation efficiency by rainbow trout (*Oncorhynchus mykiss*)[a]

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Abstract

This study examined the utilisation efficiencies of three diets when fed to rainbow trout in a 28-day growth study. Fish of 96.4 ± 1.7 g (mean ± S.D.) were kept in freshwater at 13.9 ± 0.2°C. Each of the diets was fed at one of three ration levels and an additional starved treatment was also included. The diets differed by an increasing concentration of lupin (*L. angustifolius* cv. Myallie) kernel meal (MKM) inclusion. Two lupin kernel meal inclusion levels of 15% (MKM15%) and 30% (MKM30%) were studied. The diets were formulated to equivalent digestible protein and energy specifications based on predetermined digestibility values for each of the ingredients used. However, a significantly higher level of digestible energy of both MKM diets was measured, as well as a significantly higher level of digestible phosphorus in the MKM30% diet. There were no significant differences in digestible protein level among the diets. No significant differences between the diets were observed with respect the utilisation of dietary digestible energy. Over the full data range, the energy utilisation efficiency was described by the linear equation of; $y = 0.747x - 26.174$, $R^2 = 0.985$. Efficiency of protein utilisation over lower digestible protein intake levels was also linear ($y = 0.599x - 0.142$, $R^2 = 0.905$), but over the full range was better described by a non-linear function. The comparison of the three diets in this study shows that the dietary inclusion of lupin kernel had no significant effect on the gain of either protein or lipid energy relative to protein or lipid energy intake, respectively. Protein energy use efficiency constants varied depending on the feed intake level, but were not significantly affected by diet type. The efficiency of use of lipid energy for lipid energy retention was also not affected by diet type. The findings of this study demonstrate that the inclusion of lupin kernel meal does not affect the ability of rainbow trout to utilise the dietary digestible protein and energy of diet in which it is included.

10.1 Introduction

Lupin (*Lupinus* spp.) meals have been shown to provide some potential as a useful feed ingredient in fish diets and are being used in commercial diets in increasing quantities (Burel et al., 1998; Glencross and Hawkins, 2004). There are traditionally three lupin species that are commercially produced and used as feed ingredients. These are the European white lupin

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(*Lupinus albus*), the Australian narrow-leafed lupin (*Lupinus angustifolius*) and the yellow lupin (*Lupinus luteus*) (Gladstones, 1998; Petterson, 2000). Typically it is the kernel meals of lupins that are being used in fish diets. This is supported by numerous reports on the nutritional evaluation of each of the three lupin kernel meal varieties in aquaculture diets (De la Higuera et al., 1988; Gomes et al., 1995; Burel et al., 2000; Farhangi and Carter, 2001; Glencross and Hawkins, 2004; Glencross et al., 2004; Glencross et al., 2005).

However some problems with high inclusion levels of lupins in fish diets have been reported, with minor aberrations in digestion, growth and metabolic processes (Burel et al., 1998; Farhangi and Carter, 2001; Glencross et al., 2004). These have been attributed to a range of issues including some possible anti-nutritional factors (Francis et al., 2001; Glencross et al., 2003; Glencross et al., 2006). In other studies a decline in growth has been noted with progressive inclusion of lupin, although it has been argued that this may be the result of variability in digestible or utilisation value of the diets with increasing inclusion level of lupin (Farhangi and Carter, 2001; Glencross et al., 2004).

One way of resolving whether lupin use actually affects the utilisation value of diets is to examine the protein and energy utilization values of a series of diets using a bio-energetic approach (Cho and Kaushik, 1990; Kaushik and Medale, 1998). In this sense the efficiency with which dietary protein and energy are used for growth with varying feed intake levels can be used to discern the discrete nutritional value of a diet (Lupatsch et al., 2003). By comparing several diets, the relative protein and energy utilisation efficiency among the diets can be used to discern the discrete value of each diet and by inference its formulation variable. The advantage of such an approach is that by comparing regressed utilisation values, effects of variable intake or differences in digestible value of the diets can be countered and considerable experimental power gained (Searcy-Bernal, 1995).

This study reports on the evaluation of the bio-energetic utilisation value of lupin (*Lupinus angustifolius*) kernel meals when fed to rainbow trout, *Oncorhynchus mykiss*. The study compares the effect of the inclusion of this ingredient in fish diets on the relative effects it creates on the nutrient and energy utilisation efficiency of diets compared to a diet where fish meal is the sole protein source. In particular it provides a succinct assessment of the hypothesis that fish can utilise the protein content of some grain sources as effectively as they can use fishmeal protein, provided diets are prepared on a digestible nutrient basis and are neither nutrient nor energy limiting.

10.2 Methods

10.2.1 Ingredients and diet preparation

Composition and source of all of the ingredients used is presented in Table 10.1. Lupin kernel meal (*Lupinus angustifolius,* cv. Myallie) was obtained from commercial grain millers and ground to < 600µm particle size. The remaining feed ingredients were obtained as detailed in Table 10.1.

All experiment diets were formulated to be isonitrogenous (400 g/kg) and isoenergetic (18.0 MJ/kg) on a digestible nutrient/energy basis. Digestibility coefficient values for key ingredients were based on those reported earlier (Glencross et al., 2005a). Diets were processed by the addition of water (about 30% of mash dry weight) to all ingredients while mixing to form a dough, which was subsequently screw-pressed through a 4 mm diameter die using a pasta maker. The resultant moist pellets were then oven dried at 70°C for approximately 24 h before being air-cooled, bagged and stored at -20°C. Formulations and proximate composition for all diets are presented in Table 10.2.

10.2.2 Fish handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (250 L). Freshwater (salinity < 1 PSU; Dissolved oxygen 9.6 ± 0.5 mg/L, mean ± S.D.) of 13.9 ± 0.2°C (mean ± S.D.) at a flow rate of about 4 l/min was supplied to each of the tanks. Each of the tanks were stocked with 20 trout of 96.4 ± 1.7 g (mean ± S.D.; n = 240). Photoperiod was maintained at 12:12 (light:dark). Treatments were randomly assigned amongst 30 tanks, with each treatment having three replicates. For all weight assessments the fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness.

The fish were fed to four levels of feed intake ranging from a starved treatment to apparent satiety and two intermediary feed levels, once daily at 0800h for 28 days. Apparent satiety was determined by a loss in feeding activity, this was reached after three feeding sessions over a one-hour period. Any uneaten feed was removed from each tank one hour later and the uneaten portion dried and weighed to allow the determination of daily feed intake based on correction factors for leaching losses sustained over an equivalent period (Helland et al., 1996).

Fish were individually re-weighed after four weeks, with all fish within each tank used to determine the average weight gain/loss per tank and treatment (Table 10.3). Five fish were taken as an initial sample for composition analysis. At the end of the study three fish were taken from each tank for whole body analysis. Growth was assessed as mean weight gain and thermal growth coefficient (TGC). TGC was calculated as (Kaushik, 1998):

$$TGC = \frac{\left(W_f^{1/3} - W_i^{1/3}\right)}{time \times mean\,temperature} \times 100$$

Faeces were also collected at the end of the study following their final weighing, from the satietal fed treatments. The stripping techniques used were based on those reported by Austreng (1978). The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not contaminated by urine and mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial on ice and later stored in a freezer at -20°C. Faecal samples kept frozen at -20°C before being freeze-dried in preparation for analysis.

10.2.3 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, ytterbium, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total ytterbium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by Hillebrand et al. (1953). Protein levels were calculated from the determination of total nitrogen by LECO analyser Dumas method, based on N x 6.25. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the Soxhlet method. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Given that the protein, fat and energy values from

the same samples were determined (n=30) it was decided to determine the energy equivalents for protein and fat directly from the composition of the fish tissues (Lupatsch et al., 2003). These energy equivalents were determined derived from multiple regression based on:

Energy gain (kJ) = *a* x protein gain (g) + *b* x lipid gain (g)

Using multiple regression methods the energy equivalents were determined as: for protein 20.91 ± 3.75 kJ/g and for lipid 36.33 ± 2.98 kJ/g (mean ± S.D.). These values were used in determining the energy partitioning value associated with the gain of each nutrient type.

Differences in the ratios of the parameters of dry matter, protein or gross energy to ytterbium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Yb_{diet} \times Parameter_{faeces}}{Yb_{faeces} \times Parameter_{diet}} \right)$$

where Yb_{diet} and Yb_{faeces} represent the ytterbium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively. Digestible nutrient and energy values for each diet are presented in Table 10.2.

10.2.4 Protein and energy retention

Protein (N) and Energy (E) retention were determined based on the mass gain in both N and E over the course of each block, against the respective consumption of N and E. Both values were calculated according to the following formula (Maynard and Loosli, 1969):

$$Nitrogen\ Retention = \left(\frac{Nt - Ni}{Nc} \right) \times 100$$

Where *Nt* is the nitrogen content of the fish in a specific replicate at time *t* and *Ni* is the initial nitrogen content of the fish from the beginning of the study (n=3 replicates of 3 representative fish). *Nc* is the amount of nitrogen consumed by the fish from the time of initial assessment to time t. Determination of Energy retention was achieved the same way, but with the substitution of the relevant energy criteria where the corresponding nitrogen criteria are indicated in the equation. In this study these values are determined based on gross nitrogen and energy intake only.

To provide some independence of size effects, modeling of the protein and energy retention efficiency data was done with respect to known energy and protein body-weight exponents for rainbow trout of $x^{0.8}$ and $x^{0.7}$ respectively (Cho and Kaushik, 1990).

10.2.5 Statistical analysis

All figures are mean ± SE unless otherwise specified. Effects of diets and ration levels were examined by MANOVA using the software package Statistica (Statsoft®, Tulsa, OA, USA). Levels of significance were determined using Fishers LSD test for planned comparisons, with critical limits being set at $P < 0.05$. Multiple regression analysis to determine energy equivalents was also done using Statistica. Regression figures presented were constructed using Microsoft Excel.

10.3 Results

10.3.1 Diet digestibility

Significant differences between the digestibilities of the reference and MKM diets were determined. A significantly higher level of digestible energy of both MKM diets was measured, as well as a significantly higher level of phosphorus digestibility in the MKM30% diet, but not total digestible phosphorus, which was maintained at around 10 g/kg DM in all three diets. There were no significant differences in digestible protein level among the diets (Table 10.2).

10.3.2 Energy utilisation

Efficiency of energy utilisation over lower digestible energy intake levels was linear (R^2 = 0.9849), but over the full range was better described by a non-linear function (R^2 = 0.9894). No significant differences between the diets were observed with respect the utilisation of dietary digestible energy (Figure 10.1). Over the full data range and for all treatments the energy utilisation efficiency was described by the linear equation of: $y = 0.7473x - 26.174$, $R^2 = 0.9849$. There were no significant differences among the diets in the energy utilisation efficiency determined over this data range. However, over the lower linear range the energy utilisation efficiency was described by the linear equation of: $y = 0.8828x - 36.098$, $R^2 = 0.9589$. The quadratic function to describe the relationship over the full data range was: $y = -0.0007x^2 + 0.9961x - 39.296$, $R^2 = 0.9894$. There were no significant differences among the diets in the energy utilisation efficiency determined over this data range. Maintenance digestible energy intake for each diet was calculated using linear regression between the starved and lowest feed ration treatment, as being at 40.9 ± 0.57 kJ/ $kg^{0.8}$/d and did not differ significantly among the diets (range 40.5 to 41.9 kJ/$kg^{0.8}$/d).

10.3.3 Protein utilisation

Efficiency of protein utilisation over lower digestible protein intake levels was linear, but over the full range was better described by a non-linear function. Over the full data range the protein utilisation efficiency was described by the quadratic equation of: $y = -0.0351x^2 + 0.6946x - 0.1889$, $R^2 = 0.9815$. The linear equation over the same range was: $y = 0.4661x + 0.0422$, $R^2 = 0.9724$. Over the lower range of protein intake the protein utilisation efficiency was described by the linear equation of: $y = 0.5994x - 0.1422$, $R^2 = 0.9051$. No significant differences between the diets were observed with respect the utilisation of dietary digestible protein (Figure 10.2). Maintenance digestible protein intake for each diet was calculated using linear regression between the starved and lowest feed ration treatment, as being at 0.30 ± 0.012 g/ $kg^{0.7}$/d and did not differ significantly among the diets (range 0.27 to 0.34 g/$kg^{0.7}$/d).

10.3.4 Phosphorus utilisation

Without ascribed values for exponents of phosphorus metabolism an exponent of 1 was used. Efficiency of phosphorus utilisation over lower digestible phosphorus intake levels was linear, but over the full range was better described by a quadratic function. Significant differences between the diets were observed with respect the utilisation of dietary digestible phosphorus, but only at the highest feed intake levels, with diet MKM15% having significantly better phosphorus gain at the highest ration levels (Figure 10.3). Over the lower linear range the phosphorus utilisation efficiency was described by the linear equation of: $y = 0.5724x - 0.0069$, $R^2 = 0.937$. Maintenance digestible phosphorus intake for each diet, when calculated using

linear regression between the starved and lowest feed ration treatment, showed a significant difference in maintenance digestible phosphorus intake between the MKM30% diet (0.0096 µg/kg/d) and the other two diets (Fishmeal reference: 0.0137 µg/kg/d and MKM15%: 0.0141 µg/kg/d). However, based on the fitted quadratic functions there were no significant differences in maintenance digestible phosphorus intake levels among the diets.

10.3.5 Energy expenditure for deposition of protein and lipid

Because energy retention consists almost exclusively of protein or lipid deposition, the efficiency of protein and lipid gain can be considered separately using multiple regression analysis as described first by Kielanowski (1965) and more recently by Lupatsch et al. (2003). Based on either protein and lipid gain (kJ/kg$^{0.80}$ /day respectively), expressed relative to the digestible energy intake for each diet, at each ration level (Figure 10.4 and Figure 10.5 respectively), the energy partitioning value of each diet can be determined. The comparison of the three diets in this study shows that the inclusion of lupin kernel made no significant effect on the gain of either protein or lipid relative to digestible energy intake. However, it was observed that the function of the relationship between protein energy gain and protein energy intake, and fat energy gain and fat energy intake differed. In contrast to all of the other energy intake based relationships examined in this study, fat energy gain responded linearly over the entire digestible energy intake range, whereas protein gain was curvilinear (fitted as a quadratic function) relative to digestible energy intake.

The efficiency of use of protein energy for protein energy retention was consistent with the protein intake and protein deposition relationship in that it too was not a linear relationship over the full range examined in this study (Figure 10.4). To determine the constants of efficiency of use of digestible protein energy for protein energy gain, linear regression was used at either extremes of the range of the data. Protein energy use efficiency constants (1/ k_P), for each of the diets, at the lower protein energy intake level ranged between 1.56 (k_P = 0.64) and 1.59 (k_P = 0.63) and at the upper protein energy intake level ranged between 2.15 (k_P = 0.46) and 2.30 (k_P = 0.44).

The efficiency of use of lipid energy for lipid energy retention was a linear relationship over the full range examined in this study (Figure 10.5). Linear regression was used to determine the constants of efficiency of use of dietary lipid energy for lipid energy gain. Lipid energy use efficiency constants (1/ k_L) for each of the diets ranged between 0.83 (k_L = 1.20) and 0.86 (k_L = 1.16).

10.4 Discussion

This comparison of the utilisation efficiencies of key nutrients and energy from diets with varying levels of lupin kernel meal provides sound evidence of the nutritional value of this ingredient as a dietary ingredient for salmonids. The effects seen, by the inclusion of the lupin kernel meal, show that this ingredient does not have any negative impacts on key nutrient or energy utilisation by this animal. This work shows that provided nutrient and energy intake effects are considered on a digestible basis, then the utilisation of the protein and energy from a grain protein resource, like lupin kernel meal, is no poorer than that obtained from fish meal.

10.4.1 Effects of lupin meal on digestible value of diets

Despite all three diets being formulated to provide the same digestible protein and energy characteristics, a significantly higher digestible energy content of the two lupin kernel meal

(MKM) diets was measured. This observation provides some indication of the non-additive effects of formulating with grain protein meals, in this case a positive benefit. Reasons for this discrepancy are not clear, but perhaps indicate improved utilisation of other dietary components, such as lipids, by fish when fed diets containing lupins meals. This is consistent with observations by other workers studying the application of lupin kernel meals in salmonid diets (Refstie, Pers. Comm.).

The improved phosphorus digestibility of the diet with the highest lupin kernel meal inclusion is consistent with what has been observed from the application of lupin kernel meals in salmonid diets from other studies (Burel et al., 1998; Glencross et al., 2005).

10.4.2 Effects of lupin kernel meal on energy utilisation

The use of plant protein products in aquaculture diets is generally limited by the densities of digestible protein and energy in the products. In the present study it is demonstrated that lupin kernel meal can be easily included in diets at up to 30% inclusion without detriment to the diets performance. The efficiency of energy utilisation (i.e. the ratio of energy gain as a function of DE intake) is consistent among each of the treatments $k_E = 0.74$. Minor, but non-significant differences in maintenance energy demands were observed among the different diets. This energy efficiency is substantially higher than that observed in other studies on rainbow trout, where the utilisation of DE for gain (k_E) was 0.61 regardless of feeding level as well as temperature (Azevedo et al., 1998) or $k_E = 0.68$ in another study (Rodehutscord and Pfeffer, 1999). This higher energy utilisation efficiency difference is suggested to be a genetic effect, with faster growth noted previously being from the Pemberton strain of rainbow trout compared to other rainbow trout strains (Glencross et al., 2002; Molony et al., 2004). In particular, from the present study it was also noted that the growth rates (thermal growth units; Table 10.3) of the fish in this study were substantially higher than those of the study by Azevedo et al. (1998), despite being run within the temperature range covered by their study, although with much larger fish.

At the upper levels of energy intake in the present study, marginal departure from linearity was observed in the relationship between energy gain and energy intake. This contrasts much of that reported by other workers (Azevedo et al., 1998; Rodehutscord and Pfeffer, 1999). Notably, the feed intake levels and growth achieved are much greater it the present study and this difference may be a contributing factor to this effect. However the effect is consistent with presented data for *Sparus aurata*, which also clearly shows a declining efficiency in energy retention with higher energy intake levels (Lupatsch et al., 2003).

The energy retention as protein and lipid retention was estimated based on their determined energy equivalents. These energy equivalents are slightly lower than those reported by Lupatsch et al. (2003), but consistent with data that shows that fish protein levels estimated as N x 6.25 are in fact overestimates and would be more accurately reflected by N x 6.0 (Petterson et al., 1999). The calculated energy cost as DE (kJ) for each nutrient from each diet was very similar supporting further that protein from grain protein sources is not used less efficiently than that of fishmeal protein. The protein utilisation efficiency values ($1 / k_P$) determined in the present study ranged from = 1.56 to 1.59 kJ per kJ of protein energy deposited. This was marginally more efficient than that determined by Lupatsch et al. (2003) for three marine fish species (*Sparus aurata*, *Dicentrarchus labrax* and *Epinephelus aeneus*: range 1.79 to 1.90) and in carp (*Cyprius carpio*) at 1.78 (Schwarz and Kirchgenner, 1995). The energy cost ($1 / k_L$) for lipid gain was lower throughout and ranged from 0.83 to 0.86 kJ per kJ of lipid deposited.

This was substantially lower than that reported by Lupatsch et al. (2003) for the same three marine species. In carp the efficiency was estimated at 1.39 (Schwarz and Kirchgenner, 1995), demonstrating that lipid accumulation from lipid energy intake was more efficient in rainbow trout. Indeed, the values below one suggesting that lipid synthesis is being actively achieved from other substrates.

In the present study differences in protein and lipid deposition together with differences between k_P and k_L values lead to a changing contribution to the overall energy efficiency k_E. Although Lupatsch et al. (2003) anticipated that this might be the case; they did not report this in any of the three species they studied.

10.4.3 Effects of lupin kernel meal on protein utilisation

Utilisation of dietary protein by the fish in the present study differs from that of other studies in that the relationship between protein intake and protein gain is curvilinear, whereas in other studies it has been linear over the full range studied (Lupatsch et al., 2001). The primary feature of the relationship in the present study that might explain this difference in linearity is that in the present study the feed intake and therefore protein intake by the fish is substantially higher. Over the protein intake range studied by Lupatsch et al. (2001), the relationship is also linear, with a deterioration in efficiency only seen above a protein intake of 2 g/ $kg^{0.7}$ /d. That the protein utilisation efficiency did not differ between diets at any part of the protein intake range supports that lupin protein is being used as effectively as fishmeal protein in supporting growth of the trout. In the linear range of the relationship, the determined protein utilisation efficiency of 0.60 from the present study is marginally higher than the value of 0.52 reported by Lupatsch et al. (2001) for *Dicentrarchus labrax*.

The responses seen between digestible protein intake and protein gain are also consistent with the protein energy use by the fish in this study. As with utilisation of digestible protein by the fish, the relationship between protein energy intake and protein energy gain is also curvilinear. This is also somewhat consistent with some of the observations by other workers on *Sparus aurata* but not *Dicentrarchus labrax* and *Epinephelus aeneus* (Lupatsch et al., 2003). In that study *Sparus aurata* also showed curvilinear relationship between protein energy intake and protein energy accretion. Generally the use of protein from the diets in the present study is consistent with what is known from vertebrates, that the synthesis of protein is less efficient than the synthesis of lipids (Klein and Hoffmann, 1989; Lupatsch et al., 2003). It has been suggested that in growing fish that the protein turnover exceeding protein synthesis is the main reason for a relatively low energy efficiency for protein deposition (Meyer-Burgdorff and Rosenow, 1995). This would be consistent with comparative observations on net protein turnover in the gastrointestinal tract of pigs, poultry and fish (Simon, 2002).

10.4.4 Effects of lupin kernel meal on phosphorus utilisation

The assessment of the utilisation of phosphorus in this format has little to compare with in other published studies. The relationship was not as well defined as that of protein or energy, perhaps being more subject to error in assessment because of its inherent low levels in both the feeds and fish. Irrespective, no significant differences were observed in the efficiency of phosphorus use at the lower levels of phosphorus intake. Interestingly, at the higher intake levels a significant improvement in phosphorus retention was noted from the MKM15% diet, but not the MKM30% diet.

It would be of value to revisit this assessment once the exponent of phosphorus metabolism has

been identified. In the present study an exponent of 1.0 has been assumed, in contrast to 0.7 for protein metabolism and 0.8 for energy metabolism.

10.4.5 Conclusions

The results from this study show that provided diets are formulated on a digestible nutrient and energy basis, then the inclusion of lupin kernel meal in a diet for rainbow trout does not negatively affect the ability of the animal to utilise nutrients or energy from that diet. This is an important finding which demonstrates a sound ability of these animals to utilise plant protein resources as effective ingredients to an equivalent capacity as is achieved from animal protein resources such as fish meals.

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Tables and Figures

Table 10.1 Nutrient composition of the ingredients used in the studies (all values are g/kg DM unless otherwise indicated).

Nutrient	[a]Fish meal	[a]Fish oil	[b]Pregelled wheat starch	[c]Cellulose	[d] MKM
Dry matter content (g/kg)	917	990	906	933	908
Crude protein	770	0	7	3	466
Crude fat	68	970	11	2	83
Ash	142	20	3	2	34
Phosphorus	22	0	0	0	6
Crude Fibre	11	0	2	762	37
Gross energy (MJ/kg DM)	21.3	21.3	17.2	17.3	20.7
Arginine	43	–	0	0	42
Histidine	25	–	0	0	9
Isoleucine	28	–	2	0	15
Leucine	55	–	0	0	26
Lysine	46	–	1	0	11
Methionine	21	–	0	0	2
Phenylalanine	29	–	0	0	14
Threonine	32	–	2	0	14
Valine	34	–	0	0	14

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia.

[b] Supplied by Weston BioProducts, Henderson, Western Australia, Australia.

[c] Supplied by ICN Biomedical, Costa Mesa, CA, USA.

[d] MKM: *L. angustifolius* (cv. Myallie) kernel meal supplied by Coorow Seed Cleaners, Coorow, Western Australia, Australia

Table 10.2 Formulations and composition of the experiment diets.

	Reference	MKM-15%	MKM-30%
Ingredient (g/kg)			
Ytterbium oxide	1	1	1
Pre-mix vitamins*	5	5	5
Cellulose	151	94	37
Pregelled starch	50	50	50
Fish oil	144	149	154
Fish meal	649	551	453
L. angustifolius kernel meal	0	150	300
Composition as Determined (g/kg DM)			
Dry matter content (g/kg)	952	947	947
Crude protein	483	479	476
Digestible protein	434 ± 0.9[a]	433 ± 2.1[a]	427 ± 0.9 [a]
Crude fat	210	215	231
Ash	109	98	89
Phosphorus	20	18	18
Crude Fibre	82	30	41
Gross energy (MJ/kg DM)	23.6	23.9	24.5
Digestible Energy (MJ/kg DM)	17.6 ± 0.23 [a]	18.5 ± 0.22 [b]	18.4 ± 0.31 [b]
Arginine	32	33	33
Histidine	11	10	10
Isoleucine	19	18	17
Leucine	32	31	29
Lysine	34	31	28
Methionine	12	10	9
Phenylalanine	17	16	15
Threonine	17	16	15
Valine	5	5	5

* Vitamin and mineral premix sourced from Aventis Animal Nutrition, Goodna, Queensland, Australia: includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Different superscripts, where applicable, indicate significant differences at $P < 0.05$.

Table 10.3 Growth performance, composition and nutrient retention (mean, n=3) of fish fed the experimental diets.

	Starved	Reference			MKM – 15%			MKM – 30%			Pooled SEM
		L	M	H	L	M	H	L	M	H	
Fish performance criteria*											
Initial weight (g)	96.0	96.0	97.7	94.1	95.3	98.0	94.9	96.5	96.6	97.3	0.25
Final weight (g)	86.7 [a]	110.9 [b]	136.3 [c]	200.5 [d]	110.3 [b]	136.1 [c]	211.4 [d]	114.0 [b]	136.7 [c]	220.8 [d]	5.86
Gain (g)	-9.3 [a]	14.9 [b]	38.5 [c]	106.4 [d]	15.0 [b]	38.1 [c]	116.6 [d]	17.5 [b]	40.1 [c]	123.5 [d]	5.91
TGC * (%/C°d)	-0.04 [a]	0.06 [b]	0.14 [c]	0.34 [d]	0.06 [b]	0.14 [c]	0.36 [d]	0.07 [b]	0.14 [c]	0.37 [d]	0.041
Survival (%)	100	100	100	100	100	100	100	100	100	100	0.00
Nitrogen retention (%)	0.0 [a]	42.9 [bcd]	42.6 [bcd]	37.6 [c]	37.9 [bc]	42.7 [bd]	38.4 [bc]	44.5 [d]	41.3 [bcd]	37.8 [bc]	1.01
Energy retention (%)	0.0 [a]	22.4 [b]	42.9 [c]	46.8 [d]	20.3 [b]	39.2 [c]	45.1 [d]	24.9 [b]	37.8 [c]	47.9 [d]	1.75
Phosphorus retention (%)	0.0 [a]	18.1 [b]	42.7 [d]	23.9 [b]	19.7 [b]	44.9 [d]	29.2 [c]	33.6 [c]	49.1 [d]	24.7 [b]	2.73
Feed intake (g/fish)	0.0 [a]	16.2 [b]	32.4 [c]	90.5 [d]	16.2 [b]	32.4 [c]	97.7 [d]	16.2 [b]	32.4 [c]	98.6 [d]	4.71
FCR ** (g:g)	0.000 [a]	1.09 [b]	0.84 [c]	0.85 [c]	1.09 [b]	0.85 [c]	0.84 [c]	0.93 [bc]	0.81 [c]	0.80 [c]	0.044

* TGC: Thermal Growth Coefficient.
** Food Conversion Ratio; grams of dry matter consumed per grams live-weight gain.
***Different superscripts indicate significant differences within rows at $P < 0.05$. Absence of superscripts in any one row indicates that there were no significant differences among treatments for that parameter.

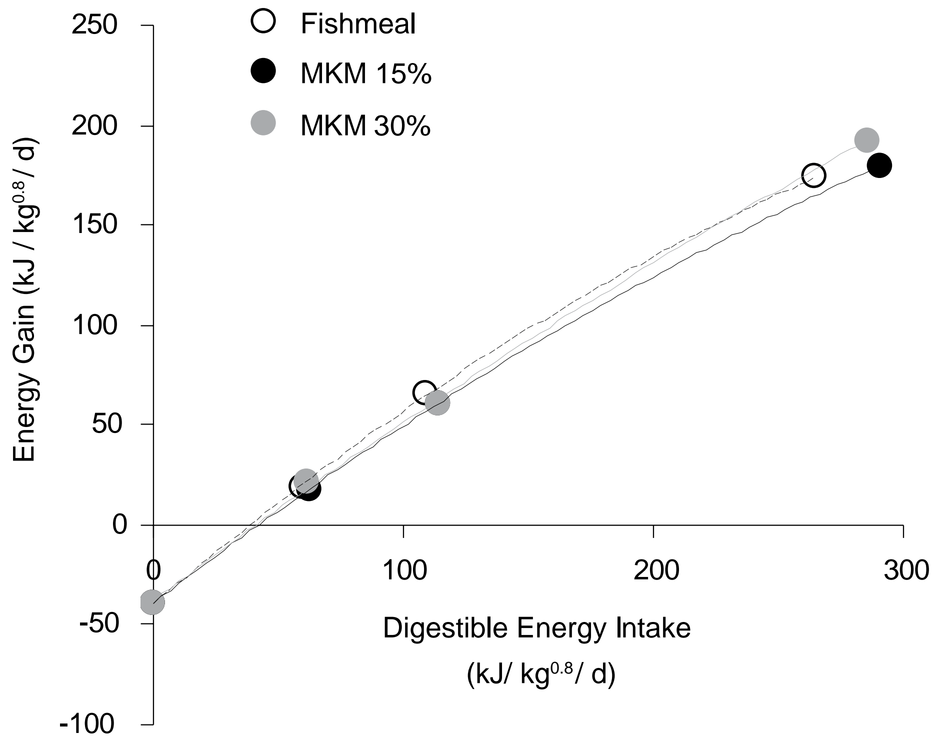

Figure 10.1 Energy gain with varying levels of digestible energy intake for each treatment.

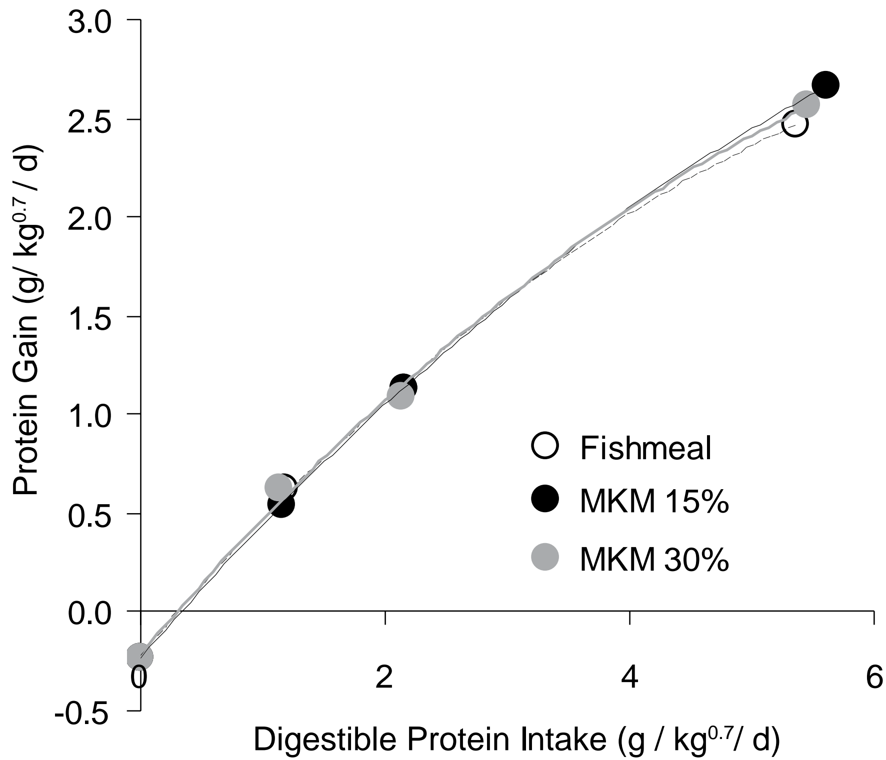

Figure 10.2 Protein gain with varying levels of digestible protein intake for each treatment.

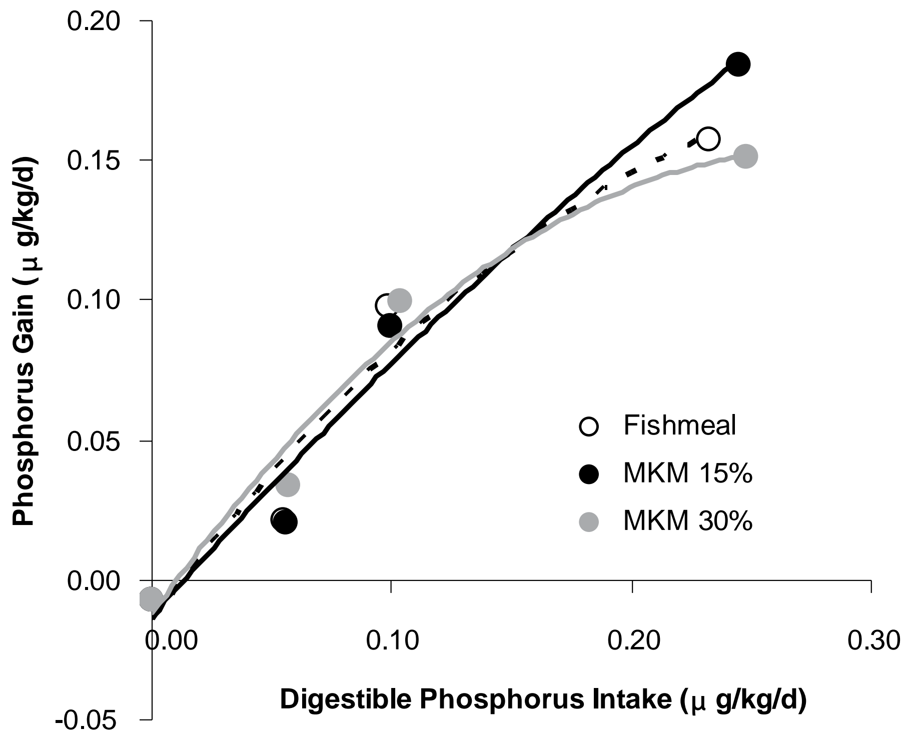

Figure 10.3 Phosphorus gain with varying levels of digestible phosphorus intake for each treatment.

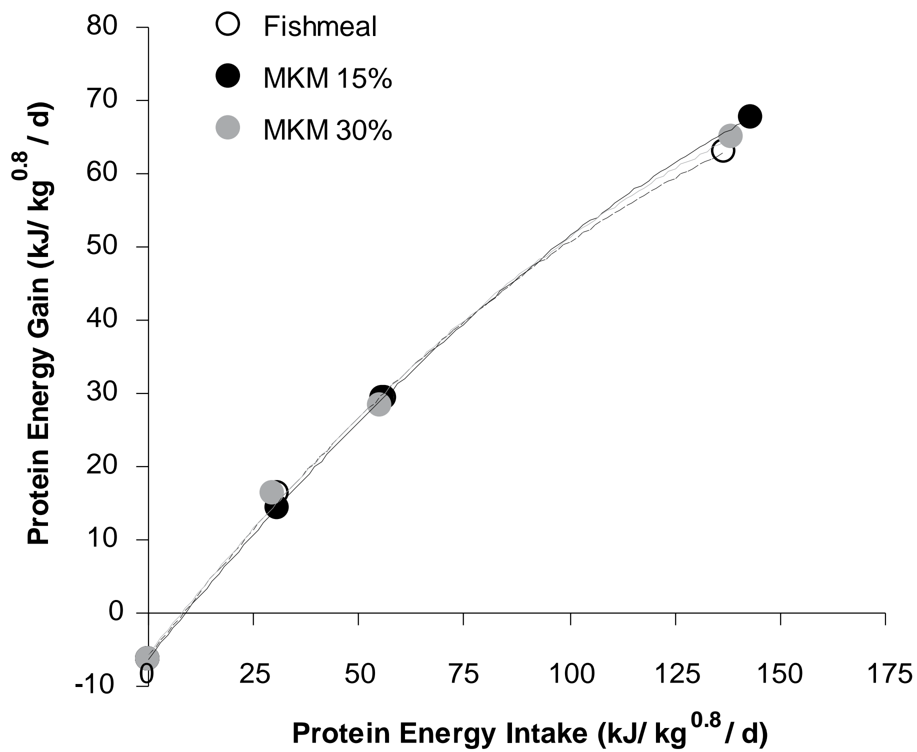

Figure 10.4 Protein energy retention with varying levels of digestible protein energy intake for each treatment.

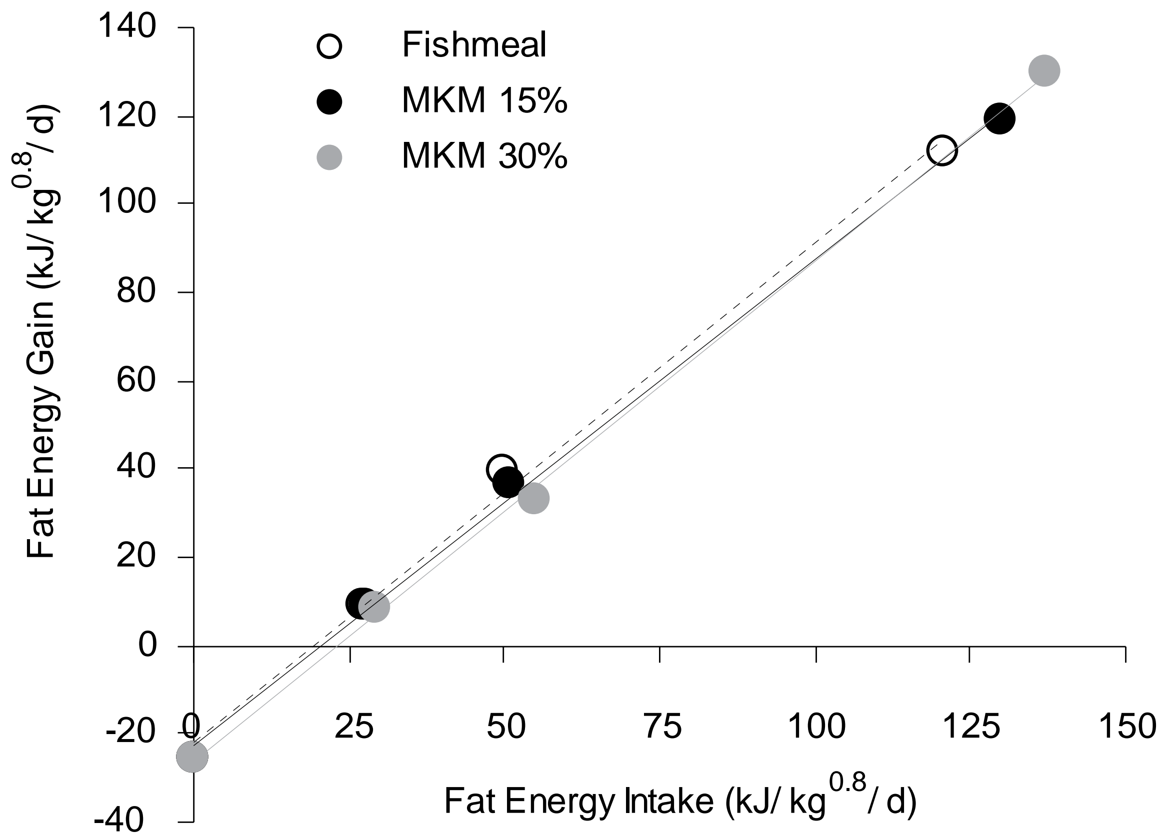

Figure 10.5 Fat energy retention with varying levels of crude fat energy intake for each treatment.

11.0 Assessing the implications of variability in the digestible protein and energy value of lupin kernel meals when fed to rainbow trout, *Oncorhynchus mykiss*[a]

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Abstract

A series of studies were designed to examine the degree of variability in the digestibility of protein and energy from lupin *(Lupinus angustifolius)* kernel meals when fed to rainbow trout (*Oncorhynchus mykiss*) and the potential implications of this variability. The digestibility of protein and energy from 10 different varieties of lupin kernel meal was assessed using the diet-replacement ingredient assessment method, where the test ingredient comprised 30% of each test diet. Digesta was collected using faecal stripping techniques. From these initial digestibility studies significant differences in protein and energy digestibilities were determined from different lupin kernel meal samples. Digestible protein value ranged from 331 to 508 g/kg DM and digestible energy values ranged from 10.6 to 13.3 MJ/kg DM. To examine the implications of variability in digestible protein and energy value, two lupin kernel meals from the extremes of the protein digestibility range (Lupin-1: AD_N ~70% and Lupin-2: AD_N ~100%) were chosen for assessment in two growth studies. Soybean meal and a reference diet with fishmeal as the only protein source were also included in the study. In the first growth experiment the test ingredients were included at equal concentrations (40%) in protein-limiting diets (350 g protein/kg DM) and fed at either of two ration levels (restricted and satiety). Diets were formulated on a crude-basis so as to place the test variable on the variability in digestible protein value of the diets. In the restricted-fed treatments growth of fish fed the reference diet was highest, but not significantly better than lupin-H. Growth of fish fed the lupin-L diet was significantly poorer than both the reference and lupin-H diets. In the satietal fed fish the soybean diet had poorer growth than all other treatments, but also had the poorest feed intake. Growth of fish fed the lupin-L diet was significantly poorer than both the reference and lupin-2 diets, but not poorer than the soybean diet. The growth responses observed from this experiment clearly showed that the differences in feed intake and/or digestible protein value could be demonstrated in terms of significant differences in growth outcomes. In a second growth study high-nutrient dense extruded diets (400 g protein/kg and 23.5 MJ/kg) were prepared with a more practical level of 25% inclusion of the same test materials. Again the diets were formulated on crude basis so as to place the test variable on the variability in digestible protein and energy value of the diets. Growth of fish restrictively fed the lupin-H diet was highest, but not significantly better than the soybean, reference or lupin-L treatments. Growth of fish satietal fed the soybean diet was significantly poorer than the reference and lupin-H diets, but not the reference of or lupin-L diet. The reference diet had poorer growth than all other treatments, but the soybean diet had the poorest feed intake, while the reference diet had the greatest intake. The growth responses observed from this experiment showed that the differences in digestible protein and energy

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value could not be demonstrated in terms of significant differences in growth outcomes, and that feed intake variability and excess nutrient supply masked the effect of this variability; particularly at the satietal feed intake levels.

11.1 Introduction

Lupin (*Lupinus* spp.) meals have been shown to provide some potential as a useful feed ingredient in fish diets and are being used in commercial diets in increasing quantities (De La Higuera et al., 1988; Burel et al., 1998). The Australian narrow-leafed lupin (*Lupinus angustifolius*) dominates world production and lupin use in aquaculture diets worldwide (Glencross and Hawkins, 2004; Glencross et al., 2004a). Typically it is the kernel meals of lupins that are being used in aquaculture diets because of their greater nutritional value than whole-seed meals (Glencross et al., 2007c).

However problems with high (> 30%) inclusion levels of lupins in fish diets have been reported, including minor aberrations in digestion, growth and metabolic processes (Burel et al., 1998; Farhangi and Carter, 2001; Glencross et al., 2004b). These have been attributed to a range of issues including some possible anti-nutritional factors (Francis et al., 2001; Glencross et al., 2003b; Glencross et al., 2006). In other studies a decline in growth has been noted with progressive inclusion of lupin, although it has been argued that this may be the result of variability in digestible or utilisation value of the diets with increasing inclusion level of lupin (Farhangi and Carter, 2001; Glencross et al., 2004b). However, it has been argued that digestible energy or protein measurement of lupins is not necessarily an adequate descriptor for quality of this grain and that there is a need to assessment of animal growth responses to varying inclusion or intake levels (van Barneveld et al. 1996).

The issue of variability in nutritional value can be resolved by using a pair-fed restricted feeding approach to limit intake variability and thereby place the experimental pressure on the nutritional composition of the diet, rather than the sum this nutritional value and feed intake effects. This experimental pressure can be further enhanced by using protein-limiting diets to ensure that the diet protein content becomes a more sensitive test variable (Glencross et al., 2003c; Glencross et al., 2007a).

Another way of resolving whether lupin use actually affects the utilisation value of diets is to examine the protein and energy utilisation values of a series of diets using a bio-energetic approach (Cho and Kaushik, 1990; Kaushik and Medale, 1998; Glencross et al., 2007b). In this sense the efficiency with which dietary protein and energy are used for growth with varying feed intake levels can be used to discern the discrete nutritional value of a diet (Lupatsch et al., 2003; Glencross et al., 2007b). By comparing several diets, the relative protein and energy utilisation efficiency among the diets can be used to discern the discrete value of each diet and by inference its formulation variable. The advantage of such an approach is that by comparing regressed utilisation values, effects of variable intake or differences in digestible value of the diets can also be countered and considerable experimental power gained.

This study reports on the evaluation of the variability in the digestibility of a range of lupin kernel meals. The influence that this variability has on the overall nutritional value of the diets fed to rainbow trout, *Oncorhynchus mykiss* is then assessed in two separate experiments. Both protein-limiting and commercially equivalent diets were used to examine and the effects of the variability in digestible value of the lupin kernel meals.

11.2 Methods

11.2.1 Raw materials

Ten samples of whole-seed *L. angustifolius* cultivars were obtained from the West Australian Department of Agriculture lupin breeding program at the Wongan Hills Research Station from the 2003 crop-season. The seed from each of the ten cultivars obtained was processed to produce kernel meals from each cultivar. For processing the seed was graded according to seed size using round-holed 7mm, 6mm and 5mm sieves and each segregation, of each variety, separately split using a disc-mill dehulling unit (Department of Agriculture, South Perth, WA, Australia). The split (dehulled) segregation of each variety was then pooled prior to aspiration (air stream mediated density classification) to remove the hulls from the kernels. Any remaining seed hull fragments were manually removed to ensure a 100% pure preparation of seed kernels of each variety. The kernels were then rotor-milled (Retsch, Haan, Germany) through a 750 µm screen. The composition of all experimental diets is also presented in Table 11.1.

11.2.2 Chemical analyses

All chemical analyses were carried out by independent, NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Amino acid analysis involved the samples being hydrolysed at 110°C for 24hr in 6M HCl with 0.05% Phenol. Cysteine and cystine are derivatized during hydrolysis by the addition of 0.05% 3,3'-dithiodipropoinic acid. The acid hydrolysis destroyed tryptophan making it unable to be determined. Separation was by HPLC on a Hypersil AA-ODS 5µm column using an 1100 series Hewlett Packard HPLC system. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the method of Folch et al. (1957). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry.

11.2.3 Experiment 1 – Ingredient digestibility assessment

11.2.3.1 Ingredient and diet preparation

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 11.2). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 11.2). Diets were processed by the addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient.

11.2.3.2 Fish handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 L). Freshwater (salinity < 1 PSU, Dissolved oxygen 9.2 ± 0.50 mg/L, mean ± S.D.) of 15.9 ± 0.20°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 20 trout of 198.0 ± 33.8 g (mean ± S.D.; n = 40). Treatments were randomly assigned amongst 44 tanks, over 4 blocks with each treatment having four replicates.

Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600 each day. The fish were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by earlier studies (Glencross et al., 2005).

11.2.3.3 Digestibility analysis

Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

where Y_{diet} and Y_{faeces} represent the chromium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 11.4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 1.000 (100%) were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%. All digestibility values are presented in the form of a coefficient (i.e. 0.000 to 1.000).

11.2.4 Experiment 2 – Growth assessment using protein-limiting diets

11.2.4.1 Ingredient and diet preparation

Four experimental diets containing either soybean meal, a lupin kernel meal with low-protein digestibility and a lupin kernel meal with high-protein digestibility, were formulated to be iso-nitrogenous and iso-energetic on a crude basis. Each test ingredient was included at an inclusion level of 40 %. Diets were processed by extrusion using an APV 19:25 laboratory-scale twin-screw feed extruder. Following extrusion, the pellets were oven dried at 60°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. Following drying the pellets were vacuum infused with the formulated oil allotment (Table 11.4). The reference diet was prepared in a similar manner, but without the addition of any test ingredient. The diet complete formulations and source of all of the ingredients used is presented in Table 11.4. Composition of all experimental diets is also presented in Table 11.4.

11.2.4.2 Fish management

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*) were transferred from grow-out ponds to experimental tanks (250 L). Freshwater (salinity < 1 PSU; Dissolved oxygen 9.3 ± 0.45 mg/L, mean ± S.D.) of 15.8 ± 1.00°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 20 trout of 36.7 ± 0.83 g (mean ± S.D.; n = 800). Photoperiod was maintained at 12:12 (light:dark). Treatments were randomly assigned amongst 40-tanks, with each treatment having five replicates. For all weight assessments the fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness.

The fish were fed one of two levels of feed provision; apparent satiety and a restricted, pair-fed level, once daily at 1600h for 63 days. Apparent satiety was determined by a loss in feeding activity, this was reached after three feeding sessions over a one-hour period. Any uneaten feed was removed from each tank one hour later and the uneaten portion dried and weighed to allow the determination of daily feed intake based on correction factors for leaching losses sustained over an equivalent period (Helland et al., 1996).

Fish were individually re-weighed after nine weeks (63 days), with all fish within each tank used to determine the average weight gain/loss per tank and treatment (Table 11.3). Five fish were taken as an initial sample for composition analysis. At the end of the study three fish were taken from each tank for whole body analysis. Growth was assessed as the mean weight gain.

Faeces were also collected at the end of the study following their final weighing, from the satietal fed treatments. The stripping techniques used were based on those reported by Glencross et al (2005). The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not contaminated by urine and mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial on ice and later stored in a freezer at -20°C. Faecal samples kept frozen at -20°C before being freeze-dried in preparation for analysis.

11.2.4.3 Protein and energy retention

Protein (N) and Energy (E) retention were determined based on the mass gain in both N and E over the course of each block, against the respective consumption of N and E. Both values were calculated according to the following formula (Maynard and Loosli, 1979):

$$Nitrogen\ Retention = \left(\frac{Nt - Ni}{Nc} \right) \times 100$$

Where *Nt* is the nitrogen content of the fish in a specific replicate at time *t* and *Ni* is the initial nitrogen content of the fish from the beginning of the study (n=5 replicates of 3 representative fish). *Nc* is the amount of nitrogen consumed by the fish from the time of initial assessment to time t. Determination of Energy retention was achieved the same way, but with the substitution of the relevant energy criteria where the corresponding nitrogen criteria are indicated in the equation. In this study these values are determined both on crude/gross and digestible nitrogen and energy intake basis.

11.2.5 Experiment 3 – Growth assessment using conventional diets

11.2.5.1 Ingredient and diet preparation

Four experimental diets containing either soybean meal, a lupin kernel meal with low-protein digestibility and a lupin kernel meal with high-protein digestibility, were formulated to be iso-nitrogenous (400 g/kg) and iso-energetic (23.5 MJ/kg) on a crude/gross basis. Each test ingredient was included at an inclusion level of 25%. Diets were processed by extrusion using an APV 19:25 laboratory-scale twin-screw feed extruder. Following extrusion the pellets were oven dried at 60°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. Following drying the pellets were vacuum infused with the formulated oil allotment (Table 11.6). The reference diet was prepared in a similar manner, but without the addition of any test ingredient. The diet formulations and source of all of the ingredients used is presented in Table 11.6. Composition of all experimental diets is also presented in Table 11.6.

11.2.5.2 Fish management

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*) were transferred from grow-out ponds to experimental tanks (250 L). Freshwater (salinity < 1 PSU; Dissolved oxygen 9.4 ± 0.10 mg/L, mean ± S.D.) of 18.1± 0.45°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 20 trout of 26.8 ± 0.17 g (mean ± S.D.; n = 39 tanks, 780 individually weighed fish). Photoperiod was maintained at 12:12 (light:dark). Treatments were randomly assigned amongst the tanks, with each treatment having three replicates. For all weight assessments the fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness.

The fish were fed to four levels of feed intake ranging from a starved treatment to apparent satiety and two intermediary feed levels, once daily at 1600h for 56-days. Apparent satiety was determined by a loss in feeding activity, this was reached after three feeding sessions over a one-hour period. Any uneaten feed was removed from each tank one hour later and the uneaten portion dried and weighed to allow the determination of daily feed intake based on correction factors for leaching losses sustained over an equivalent period (Helland et al., 1996).

Fish were individually re-weighed after four weeks and again at eight weeks, with all fish within each tank used to determine the average weight gain/loss per tank and treatment (Table 11.7). Five fish were taken as an initial sample for composition analysis. At the end of the study three fish were taken from each tank for whole body analysis. Growth was assessed as the mean weight gain. Faeces were also collected at the end of the study following their final weighing, from the satietal fed treatments for use in digestibility analyses of each of the feeds.

11.2.5.3 Protein and energy retention

Protein (N) and Energy (E) retention were determined based on the mass gain in both N and E over the course of each block, against the respective consumption of N and E. Both values were calculated according to the following formula (Maynard and Loosli, 1969):

$$Nitrogen\ Retention = \left(\frac{Nt - Ni}{Nc}\right) \times 100$$

Where *Nt* is the nitrogen content of the fish in a specific replicate at time *t* and *Ni* is the initial nitrogen content of the fish from the beginning of the study (n=3 replicates of 3 representative fish). *Nc* is the amount of nitrogen consumed by the fish from the time of initial assessment to time t. Determination of Energy retention was achieved the same way, but with the substitution of the relevant energy criteria where the corresponding nitrogen criteria are indicated in the equation. In this study these values are determined both on crude/gross and digestible nitrogen and energy intake basis.

To provide some independence of size effects, modelling of the protein and energy retention efficiency data was done with respect to known energy and protein body-weight exponents for rainbow trout of $x^{0.8}$ and $x^{0.7}$ respectively (Cho and Kaushik, 1990; Azevedo et al., 1998).

11.2.6 Statistical analysis

All figures are mean ± SE unless otherwise specified. Effects of diets and ration levels were examined by two-way ANOVA using the software package Statistica (Statsoft®, Tulsa, OA, USA). Levels of significance were determined using Fishers LSD test for planned comparisons, with critical limits being set at $P < 0.05$. Multiple regression analysis to determine energy equivalents was also done using Statistica. Statistical analysis of the regression constants and coefficients was made using a Kimura Likelihood Ratio test (Haddon, 2001). Regression figures presented were constructed using Microsoft Excel.

11.3 Results

11.3.1 Ingredient composition

There was substantial variability in the composition of the 10 varieties of *L. angustifolius* kernel meal used in this study. Protein concentrations in the kernel meals ranged from 452 to 538 g/kg DM (Mean ± SD: 499 ± 23.7, CV 4.7%). Total lipid concentrations in the kernel meals ranged from 52 to 74 g/kg DM (Mean ± SD: 66 ± 7.0, CV 10.5%). Carbohydrate concentrations were largely a reciprocal of the protein content of the meals. Energy density of the kernel meals ranged from 20.18 to 20.85 MJ/kg DM (Mean ± SD: 20.52 ± 0.19, CV 0.9%). The sum of amino acids in the kernel meals ranged from 417 to 537 g/kg DM (Mean ± SD: 463 ± 33.3, CV 7.2%). The least abundant essential amino acid was methionine in all varieties. The most abundant essential amino acid was arginine (Table 11.1).

11.3.2 Diet and Ingredient digestibility

Apparent digestibilities of the diets varied among the treatments. Apparent dry matter ranged from 0.700 to 0.810. Generally there was a decline in apparent dry matter digestibilities with inclusion of the lupin kernel meals (Table 11.3). Apparent protein digestibilities of the diets

ranged from 0.888 to 0.905 and were generally increased (> 0.900) with the addition of lupin kernel meals (Table 11.3). Apparent energy digestibilities of the diets ranged from 0.789 to 0.897 and generally declined (< 0.897) with the addition of lupin kernel meals (Table 11.3).

Apparent dry matter digestibilities of the actual lupin kernel meals varied from 0.425 to 0.579 (Table 11.3). The mean ± SD apparent dry matter digestibility was 0.542 ± 0.125. This translated to a digestible dry matter variability of 392 to 534 g DM/kg diet (Table 11.3). The mean ± SD digestible dry matter of the 10 kernel meals was 497 ± 115 g DM/kg.

Apparent protein digestibilities of the actual lupin kernel meals varied from 0.655 to 1.089 (Table 11.3). The mean ± SD apparent protein digestibility was 0.914 ± 0.129. This translated to a digestible protein variability of 331 to 508 g protein/kg (Table 11.3). The mean ± SD digestible protein of the 10 kernel meals was 485 ± 131 g protein/kg diet.

Apparent energy digestibilities of the actual lupin kernel meals varied from 0.522 to 0.647 (Table 11.3). The mean ± SD apparent energy digestibility was 0.629 ± 0.105. This translated to a digestible energy variability of 10.58 to 13.35 MJ/kg (Table 11.3). The mean ± SD digestible dry matter of the 10 kernel meals was 13.18 ± 3.02 MJ/kg diet.

11.3.3 Growth assessment using limiting-constraint diets

Each of the diets used in experiment 2 had a similar level of crude protein and gross energy. However, significant differences were determined in the levels of digestible protein and energy in the diets. Diet L had significantly lower digestible protein than diet H, but not diet R or S. Digestible energy levels in diet L were significantly lower than diets R and H, but not diet S (Table 11.4).

Growth of fish was significantly affected by both feed type and ration level. Within each feed type growth was significantly less in the restricted rations in all cases (Table 11.5). Within the restricted ration class, the gain by fish fed the L-diet fed fish was significantly less than those fed the R-diet and numerically less than both the H and S-diets (Table 11.5). Feed conversion showed significant differences between the L-diet and all other diets. Retention of protein and energy between feed rations also showed some effects, with all restricted fed fish had reduced retention efficiencies compared to the satietal fed fish.

Crude protein retention was significantly reduced with feed restriction of the L-diet, but not so with any of the other diets (Table 11.5). The L-diet had significantly poorer crude protein retention when restrictively fed than all other diets, except the crude protein retention of the restrictively fed S-diet. There were no significant differences in crude protein retention when fish were fed any of the diets to satiety (Table 11.5). The L-diet showed the largest decline in crude protein retention between restricted (35.4%) and satietal (44.2%) fed regimes.

Digestible protein retention of satietal fed fish was unaffected by diet type (Table 11.5). The H-diet had significantly higher digestible protein retention when restrictively fed than the S-diet. There was a significant effect of ration on the digestible protein retention of the L-diet with the satietal fed fish (56.9%) having a significantly higher retention than the restrictively fed fish (45.5%) (Table 11.5). Consistent with the effect observed on the crude protein, the L-diet also showed the largest decline in digestible protein retention between restricted (45.5%) and satietal (56.9%) fed regimes.

Gross energy retention was reduced with feed restriction of all diets (Table 11.5). The S-diet had significantly poorer gross energy retention when satietal fed than the H- and L-diets, but not the

R-diet. However, the L-diet had significantly poorer gross energy retention when restrictively fed than the R- and S-diets, but not the H-diet (Table 11.5). The L-diet showed the largest decline in gross energy retention between restricted (25.0%) and satietal (47.9%) fed regimes.

Digestible energy retention was reduced with feed restriction of all diets (Table 11.5). The L-diet had significantly higher digestible energy retention when satietal fed than all other diets. The H-diet had significantly lower digestible energy retention when restrictively fed, than all other diets. Consistent with the effect observed on the gross energy, the L-diet also showed the largest decline in gross energy retention between restricted (40.1%) and satietal (76.8%) fed regimes.

11.3.4 Growth assessment using practical diets

Each of the diets used in experiment 2 had similar levels of crude protein and gross energy. There were no significant differences determined in the levels of digestible protein and energy in the diets. Diet-L had the lowest level of digestible protein (358 g/kg) and diet-R (390 g/kg) the highest (Table 11.6). Diet-L also had the lowest level of digestible energy (19.4 MJ/kg) and diet-H (20.8 MJ/kg) the highest (Table 11.6).

Growth of fish was significantly affected by both feed type and ration level. Within each feed type growth was significantly less with each level of feed restriction in all cases (Table 11.7). At 4-weeks, within the restricted ration classes, but across diet types there were no significant differences in growth (Table 11.7). Feed conversion was significant poorer when fish were restricted in their feed ration. This effect was observed at both the 4-wek and 8-week assessments. At the 4-week assessment the poorest FCR was that of the low-ration H-diet and the best FCR was that of the satietal fed H-diet fish (Table 11.7). At 8-weeks, the satietal fed fish showed significantly better growth when fed the H-diet, followed by the L-diet, then the S-diet and R-diet. Differences in 8-wek weight gain between the L- and S-diets were not significant (Table 11.7). Feed intake at the 8-week assessment was significantly poorer for the S-diet than the other diets. The FCR at the 8-week assessment were significantly affected by diet type, with diets R- and L- having significantly poorer FCR than diet H- and diet-S. Assessment of protein and energy retention effects is focussed on the 4-week assessments to allow comparison of both ration effects and diet types.

Crude protein retention was significantly reduced with feed restriction of each of the diets, but there was variability among diet types as the extent of this reduction (Table 11.7). Across diet types there were no significant differences in crude protein retention when fish were fed to satiety. Digestible protein retention of the satietal fed fish was significantly poorer by the R-diet fed fish (Table 11.7). The H-diet had significantly poorer digestible protein retention when fed the low ration than the R- and S-diets, consistent with the effect that was observed with crude protein (Table 11.7).

Gross energy retention was reduced with feed restriction of all diets (Table 11.7). The S-diet had the least effect of feed ration on gross or digestible energy retention. The H-diet had the greatest effect on gross and digestible energy retention with varying feed ration (Table 11.7). The L-diet had the poorest gross and/or digestible energy retention at each feed ration level (Table 11.7).

Utilisation efficiencies of gross energy were significantly poorer by fish fed the L-diet (Figure 11.1). However, utilisation efficiencies of crude protein were not significantly affected by diet type (Figure 11.2). When examined on a digestible basis there were no significant effects of diet type on either energy or protein utilisation efficiency (Figure 11.3 and 11.4).

11.4 Discussion

Nutritional quality of feed raw materials is a comparative assessment of the capacity for a specific raw material to provide certain nutrients to a particular animal while being free of chemical and physical contaminants (van Barneveld, 2001). One aspect of that quality is the variability in the nutritional value. Feed raw materials, like most biological materials, have an inherent level of variability in their nutritional value. This has important implications in diet formulation because the objective of the formulation process is to create a blend of raw materials to produce a defined product of a specific composition and nutritional value. The introduction of variability in composition or nutritional value of the raw materials introduces a source of error. To avert this potential error, formulators have to either or both increase their specification limits to ensure that any errors don't impinge on the target composition and nutritional value, or accurately measure the composition and nutritional value of each raw material prior to the formulation process. Both strategies add a cost factor to the diet manufacture process, but significantly reduce formulation risk (Jiang, 2001). An important aspect of understanding this risk is to assess the implications that such variability in raw material has on the performance of animals fed the diets (Glencross et al., 2007a). In this study the raw material variability is examined in a single ingredient, lupin kernel meals.

11.4.1 *Variability in lupin kernel meal composition*

Each of the lupin kernel meals assessed in this study had substantially higher protein content than that usually observed for lupin kernel meals (van Barneveld, 1999b; Petterson, 2000; Glencross et al., 2003a). This variability compared to other data sets is likely to be largely attributable to environmental variation because the samples were obtained from the same site from the same season (Cowling and Tarr, 2004). Accordingly, the variation within the sample set presented (Table 11.1) is solely that attributable to genotype as each of the samples.

The results show that there can be substantial variability in most composition parameters for lupin kernel meals. This finding is also consistent with other studies on other grain varieties, which show that most other raw materials show a similar degree of variability (Jiang, 2001; van Barneveld, 2001).

Variability in crude protein ranged from 452 to 538 g/kg (Table 11.1). This variability of close to 20% (between maximum and minimum) is substantial and use of standard book values could result in a significant shortfall or oversupply of protein in any formulation. To avert this risk the use of near-infrared spectroscopy (NIRS) measurement could be applied to measure the actual composition of the raw materials prior to formulation, although this has to managed through the development of appropriate calibrations (Bertrand, 2001). Variability in gross energy content was substantially less than that of the protein, reflecting the reciprocal relationship between protein and carbohydrate content in lupin kernel meals and that the energetic values of protein and carbohydrate a relatively similar. The discrepancy between the sum of amino acids and the N x 6.25 determined concentration of protein suggests that this correction factor (N x 6.25) may not be appropriate for use with lupin kernel meals.

11.4.2 *Effects of variability in lupin kernel meal digestibilities*

Each of the lupin kernel meals assessed for their digestible protein and energy value in this study were shown to have sound nutritional value. The generic protein digestibility determined across all varieties (0.914 ± 0.129, CV 14%) is consistent with other published reports on the protein digestibility of *L. angustifolius* kernel meals (Glencross and Hawkins, 2004; Glencross

et al., 2003a; Glencross et al., 2005). The generic energy digestibility determined across all varieties (0.629 ± 0.105, CV 17%) is also consistent with other published reports on the protein digestibility of *L. angustifolius* kernel meals (Glencross and Hawkins, 2004; Glencross et al., 2003a; Glencross et al., 2005). The presence of variability in digestible value of lupin kernel meals has also been previously reported (Glencross et a., 2003a). The observations in the present study are also consistent with observations by other workers studying the application of lupin kernel meals in aquaculture diets, who also observed some variability between varieties within grain species (Glencross et al., 2003a; Refstie et al., 2006; Smith et al., 2007).

The combination of variability in crude composition and that of the digestibilities was observed to be compounded, with substantially greater variability observed in the digestible value parameters. Because there is substantial variability in the values of digestible protein (CV 27%) and digestible energy (CV 23%) determined from these lupin kernel meals any means of assessing the variability in their nutritional value prior to formulation will provide reduced risk and improved viability. While it is known that there are similar levels of crude composition variability in other raw materials, it would be of value to assess whether this degree of variability in digestible protein and energy is also found in other raw materials when fed to fish (van Barneveld, 1999a; Jiang, 2001).

While use of NIRS for determining the composition of raw materials is now common in most feed production systems, the use of NIRS to assess the digestible value of protein and energy from raw materials is not as well established and remains to be successfully undertaken with any grain product in an aquaculture species (Glencross et al., 2007a). To achieve this a wide range of samples are be required from which to determine the digestible protein and energy values and to then correlate this with the NIRS spectra of the samples (Bertrand, 2001; van Barneveld et al., 1998).

11.4.3 Influence of digestible value variability in low-protein diets

The use of conventional diet formulations and feeding strategies for testing nutrient limitations is fraught with problems (Glencross et al., 2007a). Because of these problems a protein-limiting restrictively fed experiment design was used in the second experiment to enable focus to be placed on the nutritional value of the test ingredients used.

The high (40%) inclusion of the test ingredients in these experimental diets was shown to have a significant effect on both the protein and energy digestibilities of the diets (Table 11.4). Most notable was the difference between the L- and H-diets, which compared lupin kernel meals of similar composition, but known differences in digestible protein (331 vs 505 g/kg) and energy (12.3 and 12.7 MJ/kg) (Table 11.3). Ironically a bigger difference in diet digestible energy values (13.4 vs 16.3 MJ/kg) was observed, despite a smaller difference in the digestible energy values of the two kernel meals, than the difference observed between the diet digestible protein values (293 vs 335 g/kg). This supports notions of interactive effects with the inclusion of high carbohydrate materials in compounded diets.

Weight gain of fish fed the diets restrictively showed that there were clear differences in the nutritional value between the two lupin samples but that there was no significant difference between the R-, S- or H-diets (Table 11.5). Variability among replicates within the restrictively fed treatments was substantially reduced compared to the satietal fed fish. The effects of diet type were more clearly seen through the differences in the FCR of each diet at the restrictively fed levels. In this regard a higher FCR was observed for the fish fed the L-diet, significantly more so than that observed for all the other diets. This higher FCR being the combined result of

minor effects of growth and feed intake variability within this treatment and demonstrates that when feed intake is largely controlled that effects are usually observed as differences in gain or FCR (Glencross et al., 2007a). That the L-diet had significantly poorer performance when restrictively fed clearly demonstrates that the nutritional value of the lupin content of that diet is significantly poorer compared to that lupin in the H-diet. This demonstrates that it is possible to clearly determine effects of variability in digestible value of raw materials as a growth and feed utilisation response.

Growth of fish fed the diets to satiety also showed that there were clear differences in nutritional value between the two lupin samples and that even variability in feed intake with satietal feeding did not mask this difference, in fact it appeared to exacerbate it (Table 11.5). It was also observed that growth from fish fed the soybean was poorer than all other treatments and this was principally because of a reduction in feed intake compared to the other diets. This suggests that soybean introduces a palatability issue at 40% inclusion, but that lupin kernel meals do not necessarily have this problem at this same inclusion level, although feed intake by the fish fed the L-diet was also marginally reduced compared to the H- and the R-diets (Table 11.5).

The efficiencies of energy retention (i.e. the ratio of energy gain as a function of GE or DE intake) varied with both diet and feed ration level. At restricted feeding levels there was a decrease in retention efficiencies (Table 11.5). There was significant variability among the diets, with the L-diet having the highest energy retention. Considering the parabolic effects of energy retention with diminished energy intake on fish growth, these results suggest that the lower digestible energy value of the L-diet was used more efficiently at the higher intake levels because it provided a digestible energy intake closer to K_{max} than that of the other diets (Brett and Groves, 1979). Notably those retention efficiencies from the fastest growing fish (H- and R-diets) were similar to each other, but less than that of the L-diet. The substantially lower efficiencies of the restrictively fed fish are most likely because their energy intake levels were substantially lower than K_{max} (Brett and Groves, 1979).

The efficiencies of protein retention also varied with both diet and feed ration level, but not to the same degree as were observed with the energy retention efficiencies. At restricted feeding levels there was a decrease in retention efficiencies (Table 11.5). There was limited variability among the diets based on digestible protein intake, with the exception of the H-diet having significantly higher protein retention when fed restrictively and the L-diet when fed to satiety.

11.4.4 Influence of digestible value variability in normal specification diets

Although differences in nutritional value could be exhibited as growth effects when stringent experimental designs were used, to examine the practical implications of the raw material variability a third trial was conducted where the raw materials were included at more typical conservative inclusion levels and the diets were formulated to higher protein specifications.

The more conservative inclusion level (25%) of the test ingredients in these experimental diets is more consistent with the typical inclusion levels of novel ingredients in commercial formulations (Glencross et al., 2007a). Despite being included in the diets at these more conservative levels a significant effect of the raw materials being tested were observed on the growth of the fish. In contrast to the second experiment neither the protein and energy digestibilities differed significantly among the diets (Table 11.6). This is to be expected given the lower inclusion levels of the raw materials in question. Although the biggest difference in diet digestible energy values (19.4 vs 20.8 MJ/kg) was between the L- and H-diets respectively, this difference was not significant.

Weight gain of fish fed the diets restrictively (only conducted to 4-weeks) showed that there were no clear differences in nutritional value between any of the diets (Table 11.7). Variability among replicates within the restrictively fed treatments was again substantially reduced compared to the satietal fed fish, but there was insufficient variability among treatments to identify any significant effects. There were no clear effects of diet type on the FCR of each diet, although the FCR did increase with each level of feed restriction. The only exception to this was a higher FCR observed for the fish fed the H-diet at the lowest ration, which was significantly greater than that observed for all the other diets at the same ration. It is suspected that this effect, which is inconsistent with the data at the higher ration levels, is an aberration.

Growth of fish fed the diets to satiety over an 8-week period also showed that there were some subtle differences in nutritional value still observable between the two lupin samples and that even variability in feed intake with satietal feeding did not mask this difference, with a key difference being the FCR of fish fed either the H- or L-diets (Table 11.7). Consistent with experiment 2 it was also observed that growth from fish fed the soybean was poorer than all other treatments and this was principally because of a reduction in feed intake compared to the other diets. It was also noted in experiment 3 that the growth of fish fed the reference diet was less than that of fish fed either of the lupin diets (Table 11.7). The main factor affecting this appears to be a poorer conversion of the diet compared to the two lupin diets, with a poorer FCR, but higher feed intake noted.

The efficiencies of energy retention varied with both diet and feed ration level, consistent with experiment 2. At restricted feeding levels there was a decrease in retention efficiencies (Table 11.7). There was significant variability among the diets, with the L-diet having the lowest energy retention and soybean the highest with increasing levels of feed restriction. The energy retention efficiency (gross or digestible) of each of the diets when fed to satiety was similar, with only the L-diet being marginally lower than the other diets.

The efficiencies of protein retention also varied with both diet and feed ration level, but not to the same degree as were observed with the energy retention efficiencies. At restricted feeding levels there was generally a decrease in retention efficiencies, but in some cases an increase was noted (Table 11.7). When each of the diets was fed to satiety there were no differences in protein retention either on a gross or digestible basis. The lack of consistent effects with these parameters questions their value given that significant effects were noted with both weight gain and FCR among the same diets.

A further way of examining the effect of the raw materials on feed quality is to assess the efficiency of energy utilisation (i.e. the ratio of energy gain as a function of GE or DE intake, but in this case over varying intake levels) (Figures 11.1, 11.2, 11.3 and 11.4). In these assessments the gradient of the regression function coefficient is consistent with the partial utilisation efficiency of energy (k_E) or protein (k_P) as the case may be (Lupatsch et al., 2003). In the assessment of gross energy utilisation the coefficient for diets H-, S- and R- was significantly greater than that from L-diet (Figure 11.1). This supports that the lupin content of the L-diet was less efficiently utilised on a gross basis. When the same effect is examined on a digestible basis (Figure 11.3) the significant difference is lost. Although the L-diet is still marginally lower in utilisation efficiency than the other three diets. What is unusual though is the value of k_E in this case, where values of k_E =0.426 and k_E =0.375 are observed (Figure 11.3) This energy efficiency is substantially lower than that observed in other studies on rainbow trout, where the utilisation of DE for gain (k_E) was 0.61 regardless of feeding level as well as temperature (Azevedo et al., 1998) or k_E = 0.68 in another study (Rodehutscord and Pfeffer, 1999) and a k_E

= 0.74 observed from earlier work by our own laboratory (Glencross et al., 2007b). By further comparison, an analysis of the digestible energy utilisation of the diets in experiment 2 shows values of k_E= 0.70 and 0.67 from the H- and L-protein limited diets respectively both of which are more consistent with those reported in other studies.

In contrast no differences were noted from the crude protein intake (Figure 11.2) or the digestible protein intake (Figure 11.4) among any of the diets.

The effects observed from the assessment of energy and protein utilisation efficiencies support that the lupin content of the L-diet is as effectively utilised as that of the H-diet, but the key variability in its nutritional value was determined from its energy value, not its protein value.

No departure from linearity was observed in the relationship between energy gain and energy intake in this study in contrast to others conducted by our laboratory (Glencross et al., 2007b). This linearity is however consistent that reported by other workers (Azevedo et al., 1998; Rodehutscord and Pfeffer, 1999). Although the energy intake levels are similar to that in our other studies (Glencross et al., 2007b), the energy gain achieved is much lower in the present study. Similar poorer protein utilisation efficiencies were also observed in this study compared to those reported earlier (Glencross et al., 2007b). While earlier it was suggested that the differences in energy and protein utilisation efficiencies might have been a genotypic effect (Glencross et al., 2007b), we now suspect that this difference may be a dietary effect. Notably there was a substantial difference in the protein and energy balance of the diets between the two experiments.

11.4.5 Conclusions

The nutritional value of a raw material depends on both the total content and the biological availability of the specific nutrients it contains (Jiang, 2001). This biological availability has two aspects to it, the ability of an animal to absorb nutrients (digestibility) from the raw material and also the ability of the animal to convert those nutrients into growth (utilisation) (Glencross et al., 2007a). This study has demonstrated that variability in raw materials has a direct and measurable impact on their nutritional value when assessed using both digestibility and growth studies. It was also shown that this variability could be managed to a degree through increasing the diet formulation specifications to allow for an over-specification of key nutrients. However, although this formulation strategy reduces performance risk it does add a cost factor to the diet manufacturing process. The capacity to better manage this variability will depend on an improve ability to rapidly measure the nutritional value of raw materials prior to the formulation process. Adaptation of the use of near infrared spectroscopy is one of the more viable options to pursue this.

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Table 11.1 Nutrient composition of the ingredients used in the studies (all values are g/kg DM unless otherwise indicated).

					L. angustifolius kernel meals									
Actual	**Fishmeal**	**Wheat**	**Cellulose**	**Soybean**	**Wonga**	**Gungurru**	**Kalya**	**Jindalee**	**Danja**	**Yorrel**	**Tallerack**	**Mandelup**	**Coromup**	**Myallie**
Dry Matter (g/kg)	931	905	941	913	916	914	916	921	914	916	917	916	919	921
Protein (N x 6.25)	749	142	0	531	487	538	508	485	514	481	515	505	505	452
Fat	87	24	2	15	70	63	60	74	70	71	52	62	66	73
Ash	161	11	2	68	34	38	39	34	39	29	37	35	36	33
Carbohydrate [a]	3	823	996	386	409	361	392	407	377	419	397	397	393	441
Energy (MJ/kg DM)	20.52	18.43	16.98	18.94	20.62	20.62	20.54	20.63	20.47	20.85	20.18	20.53	20.44	20.26
Sum of Amino Acids [b]	670	152	9	505	448	537	494	447	473	440	443	460	466	417
Arginine	41	7	0	36	54	66	59	54	57	51	54	52	59	48
Cysteine	10	4	0	10	7	7	8	7	8	7	7	8	6	6
Histidine	13	1	0	9	9	11	11	9	11	9	9	9	10	8
Isoleucine	29	5	0	22	18	20	19	17	19	17	17	18	17	16
Leucine	56	10	0	39	33	38	36	32	35	33	32	33	34	30
Lysine	55	5	4	32	21	24	23	20	22	21	21	22	23	20
Methionine	21	2	0	7	3	3	2	3	2	2	2	3	3	2
Phenylalanine	30	6	0	24	18	21	19	17	18	17	17	18	18	16
Threonine	32	5	1	20	16	18	17	16	17	17	16	18	19	16
Valine	33	6	0	23	17	18	17	16	17	16	16	18	18	15

[b] Based on dry matter minus protein, fat and ash content.

[b] Includes all amino acids except tryptophan which was unable to be determined using the hydrolysis method used in this work.

Table 11.2 Diet formulations for experiment 1 – assessing variability in ingredient digestibilities (all values are g/kg).

Ingredient	Basal	A	B	C	D	E	F	G	H	I	J
Fishmeal [a]	700.0	490.0	490.0	490.0	490.0	490.0	490.0	490.0	490.0	490.0	490.0
Fish oil [a]	150.0	105.0	105.0	105.0	105.0	105.0	105.0	105.0	105.0	105.0	105.0
L. angustifolius cv Wonga [b]	–	300.0	–	–	–	–	–	–	–	–	–
L. angustifolius cv Gungarru [b]	–	–	300.0	–	–	–	–	–	–	–	–
L. angustifolius cv Kalya [b]	–	–	–	300.0	–	–	–	–	–	–	–
L. angustifolius cv Jindalee [b]	–	–	–	–	300.0	–	–	–	–	–	–
L. angustifolius cv Danja [b]	–	–	–	–	–	300.0	–	–	–	–	–
L. angustifolius cv Yorrel [b]	–	–	–	–	–	–	300.0	–	–	–	–
L. angustifolius cv Tallerack [b]	–	–	–	–	–	–	–	300.0	–	–	–
L. angustifolius cv Mandelup [b]	–	–	–	–	–	–	–	–	300.0	–	–
L. angustifolius cv Coromup [b]	–	–	–	–	–	–	–	–	–	300.0	–
L. angustifolius cv Myallie [c]	–	–	–	–	–	–	–	–	–	–	300.0
Wheat flour [a]	144.0	100.8	100.8	100.8	100.8	100.8	100.8	100.8	100.8	100.8	100.8
Vitamin and mineral premix [a]	5.0	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide [e]	1.0	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7

[a] Fish meal and Fish Oil - Chilean anchovy products, Wheat, Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g. : All sourced from Skretting Australia, Cambridge, TAS, Australia.

[b] Lupin kernel meals, produced from seed grown at Wongan Hills Research Station and processed at Department of Agriculture, South Perth, WA, Australia.

[c] *L. angustifolius* cv. Myallie Kernel Meal, Coorow Seed Cleaners, Coorow, WA, Australia.

[d] Supplied by SIGMA, St Louis, Missouri, United States. [d] Supplied by Stanford Materials Corporation, Aliso Viejo, CA, United States.

Table 11.3 Diet and ingredient digestibility coefficients and digestible nutrient values for each ingredient tested.

	Dry Matter	Protein	Energy
Diet digestibilities			
Reference	0.810	0.900	0.897
cv Wonga	0.706	0.900	0.797
cv Gungarru	0.718	0.900	0.808
cv Kalya	0.717	0.899	0.803
cv Jindalee	0.740	0.905	0.819
cv Danja	0.733	0.905	0.814
cv Yorrel	0.722	0.903	0.810
cv Tallerack	0.711	0.890	0.802
cv Mandelup	0.721	0.888	0.805
cv Coromup	0.713	0.897	0.803
cv Myallie	0.700	0.893	0.789
Ingredient digestibilities			
cv Wonga	0.464 [bc]	0.928 [b]	0.578 [b]
cv Gungarru	0.509 [ab]	0.919 [b]	0.601 [a]
cv Kalya	0.488 [b]	1.002 [a]	0.573 [b]
cv Jindalee	0.579 [a]	0.903 [b]	0.647 [a]
cv Danja	0.561 [a]	0.909 [b]	0.633 [a]
cv Yorrel	0.530 [ab]	0.948 [ab]	0.630 [a]
cv Tallerack	0.493 [b]	0.743 [c]	0.587 [ab]
cv Mandelup	0.527 [a]	0.655 [c]	0.597 [ab]
cv Coromup	0.492 [b]	1.083 [a]	0.624 [a]
cv Myallie	0.425 [c]	1.089 [a]	0.522 [c]
Digestible nutrient and energy levels			
cv Wonga	425	452	11.92
cv Gungarru	465	494	12.38
cv Kalya	447	508	11.76
cv Jindalee	534	438	13.35
cv Danja	513	467	12.96
cv Yorrel	485	456	13.13
cv Tallerack	452	383	11.85
cv Mandelup	483	331	12.26
cv Coromup	452	505	12.76
cv Myallie	392	452	10.58

Table 11.4 Diet formulations for experiment 2 - using limiting constraint growth studies to assess the significance of differences in ingredient digestibilities (all values are g/kg).

Ingredient	Soy	Lupin-L	Lupin-H	REF
Formulation (g/kg)				
Ytterbium oxide	1	1	1	1
CaPO4	22	16	22	10
Pre-mix vitamins	5	5	5	5
Cellulose	39	51	46	194
Fish oil	174	155	154	162
Wheat flour	150	150	150	150
Soybean meal	400	0	0	0
Mandelup kernel meal	0	400	0	0
Coromup kernel meal	0	0	400	0
Fish meal	204	215	215	478
DL-Methioine	5	7	7	0
Composition as analysed (g/kg DM unless otherwise noted)				
Dry matter	974	969	966	973
Protein	369	377	369	360
Digestible Protein	319 [ab]	293 [b]	335 [a]	319 [ab]
Fat	211	199	207	195
Carbohydrate	335	358	352	356
Phosphorus	10	10	10	10
Ash	85	66	72	89
Gross Energy	21.6	21.5	21.7	21.6
Digestible Energy	15.7 [a]	13.4 [b]	16.3 [a]	16.5 [a]
Arginine	23	17	19	25
Histidine	13	10	8	13
Isoleucine	9	8	8	9
Leucine	15	11	11	14
Lysine	25	19	19	24
Methionine	4	3	4	4
Phenylalanine	16	12	12	16
Threonine	15	10	11	13
Tryptophan	8	7	8	8
Valine	25	25	25	24

Table 11.5 Fish performance from experiment 2 – limiting constraint diets.

	Reference Diet		Lupin-L Diet		Lupin-H Diet		Soybean Diet		Pooled SEM
	Restricted	Satiety	Restricted	Satiety	Restricted	Satiety	Restricted	Satiety	
Initial weight	36.4	36.4	37.0	37.4	36.7	36.2	36.2	37.0	0.12
Weight - 9wk (g/fish)	66.8[d]	222.1[a]	64.6[e]	209.4[b]	65.0[de]	220.1[a]	64.6[e]	189.7[c]	10.91
Gain - wk 9	30.3[d]	185.6[a]	27.7[c]	172.0[b]	28.3[de]	183.9[a]	28.4[c]	152.7[c]	10.90
Feed intake - 9wk (g/fish)	33.4[d]	189.0[a]	33.5[d]	176.3[b]	32.1[d]	179.1[b]	32.1[d]	162.4[c]	10.58
FCR - wk 9	1.10[b]	1.02[a]	1.21[c]	1.02[a]	1.13[b]	0.97[a]	1.13[b]	1.06[ab]	0.20
Crude Protein Retention (%)	39.1%[b]	42.0%[b]	35.4%[c]	44.2%[ab]	45.9%[a]	47.9%[a]	38.6%[b]	44.2%[ab]	0.9%
Digestible Protein Retention (%)	45.3%[bc]	48.5%[b]	45.5%[b]	56.9%[a]	50.6%[ab]	52.8%[a]	43.5%[c]	49.9%[ab]	1.0%
Gross Energy Retention (%)	30.8%[c]	44.4%[a]	25.0%[c]	47.9%[a]	26.1%[c]	47.2%[a]	29.7%[c]	39.5%[b]	1.5%
Digestible Energy Retention (%)	40.3%[c]	58.1%[b]	40.1%[c]	76.8%[a]	34.8%[d]	62.8%[b]	40.9%[c]	54.3%[b]	2.3%

Table 11.6 Diet formulations for experiment 3 - using conventional growth studies to assess the significance of differences in ingredient digestibilities (all values are g/kg).

Ingredient	Soybean	Lupin–L	Lupin–H	R
Formulation (g/kg)				
Marker	1	1	1	1
CaPO4	4	5	5	–
Pre-mix vitamins	5	5	5	5
Cellulose	22	11	11	113
Fish oil	215	204	204	207
Wheat flour	120	120	120	120
Soybean meal	250	–	–	–
Mandelup kernel meal	–	250	–	–
Coromup kernel meal	–	–	250	–
Fish meal	383	404	404	554
Composition as analysed (g/kg DM unless otherwise noted)				
Dry matter	960	953	962	968
Protein	410	424	423	438
Digestible Protein	364 [ab]	358 [b]	372 [ab]	390 [a]
Fat	233	219	230	256
Carbohydrate	271	272	256	211
Ash	85	85	91	95
Gross Energy (MJ/kg DM)	23.3	24.3	24.8	23.8
Digestible Energy (MJ/kg DM)	19.9 [ab]	19.4 [b]	20.8 [a]	20.3 [a]

Table 11.7 Fish performance from experiment 3 – conventional growth studies.

		Reference Diet			Lupin–L Diet			Lupin–H Diet			Soybean Diet			Pooled SEM
	Starved	Low	Medium	Satiety	Low	Medium	Satiety	Low	Medium	Satiety	Low	Medium	Satiety	
Initial weight	26.9	26.9	26.7	27.0	26.7	26.8	26.9	26.7	27.0	26.7	26.8	26.7	26.9	0.02
Weight – 4wk (g/fish)	23.0 ^{a}	37.5 ^{a}	52.8 ^{a}	73.7 ^{a}	37.8 ^{a}	52.2 ^{a}	78.5 ^{a}	37.4 ^{a}	53.3 ^{a}	79.3 ^{a}	38.0 ^{a}	53.1 ^{a}	74.4 ^{a}	2.67
Gain – wk 4 (g/fish)	–3.9 ^{a}	10.6 ^{b}	26.1 ^{c}	46.8 ^{d}	11.0 ^{b}	25.4 ^{c}	51.6 ^{e}	10.7 ^{b}	26.3 ^{c}	52.6 ^{e}	11.2 ^{b}	26.5 ^{c}	47.5 ^{de}	2.67
Feed intake – 4wk (g/fish)	0.0 ^{a}	11.5 ^{b}	23.4 ^{c}	41.6 ^{d}	11.7 ^{b}	23.9 ^{c}	41.7 ^{d}	13.4 ^{b}	23.6 ^{c}	41.9 ^{d}	12.1 ^{b}	23.2 ^{c}	38.8 ^{d}	2.19
FCR – wk 4	0.00 ^{a}	1.09 ^{d}	0.90 ^{bc}	0.89 ^{bc}	1.06 ^{d}	0.94 ^{c}	0.81 ^{b}	1.25 ^{e}	0.90 ^{bc}	0.80 ^{b}	1.08 ^{d}	0.88 ^{bc}	0.82 ^{b}	0.04
Crude Protein Retention (%)	0.00 ^{a}	18.7%^{bc}	34.7% ^{d}	29.3%^{cd}	16.1%^{b}	25.1% ^{c}	32.8% ^{d}	11.9% ^{b}	29.2% ^{cd}	34.0% ^{d}	26.2% ^{c}	22.6% ^{c}	32.2% ^{d}	1.7%
Digestible Protein Retention (%)	0.00 ^{a}	21.0% ^{a}	39.0% ^{d}	32.9%^{cd}	18.7%^{b}	29.0% ^{c}	37.9% ^{d}	13.5% ^{b}	33.2% ^{a}	38.6% ^{d}	29.5% ^{a}	25.4% ^{c}	36.3% ^{d}	1.9%
Gross Energy Retention (%)	0.00 ^{a}	12.9% ^{c}	29.3%^{e}	32.6%^{e}	5.7%^{b}	20.1% ^{d}	26.3% ^{de}	7.5% ^{b}	26.8% ^{de}	32.2% ^{e}	18.6% ^{cd}	17.1% ^{c}	30.8% ^{e}	1.8%
Digestible Energy Retention (%)	0.00 ^{a}	15.2% ^{c}	34.3%^{e}	38.2%^{f}	7.1%^{b}	24.7% ^{d}	32.4% ^{e}	8.9% ^{b}	32.0% ^{e}	38.4% ^{f}	21.8% ^{d}	20.0% ^{d}	36.1% ^{ef}	2.1%
Weight – 8wk (g/fish)	–	–	–	158.4 ^{a}	–	–	166.4 ^{b}	–	–	172.2 ^{c}	–	–	162.0 ^{ab}	2.61
Gain – wk 8 (g/fish)	–	–	–	131.4 ^{a}	–	–	139.5 ^{b}	–	–	145.5 ^{c}	–	–	135.1 ^{ab}	2.61
Feed intake – 8wk (g/fish)	–	–	–	169.2 ^{a}	–	–	161.8 ^{a}	–	–	147.9 ^{b}	–	–	144.4 ^{b}	7.34
FCR – wk 8	–	–	–	1.29 ^{c}	–	–	1.17 ^{b}	–	–	1.02 ^{a}	–	–	1.07 ^{ab}	0.06
Crude Protein Retention (%)	–	–	–	26.8% ^{b}	–	–	27.8% ^{ab}	–	–	28.8% ^{a}	–	–	29.0% ^{a}	0.3%
Digestible Protein Retention (%)	–	–	–	30.1% ^{b}	–	–	32.2% ^{a}	–	–	32.8% ^{a}	–	–	32.7% ^{a}	0.3%
Gross Energy Retention (%)	–	–	–	31.8% ^{a}	–	–	31.6% ^{a}	–	–	30.7% ^{b}	–	–	32.4% ^{a}	0.1%
Digestible Energy Retention (%)	–	–	–	37.3% ^{ab}	–	–	39.0% ^{a}	–	–	36.6% ^{b}	–	–	38.0% ^{a}	0.2%

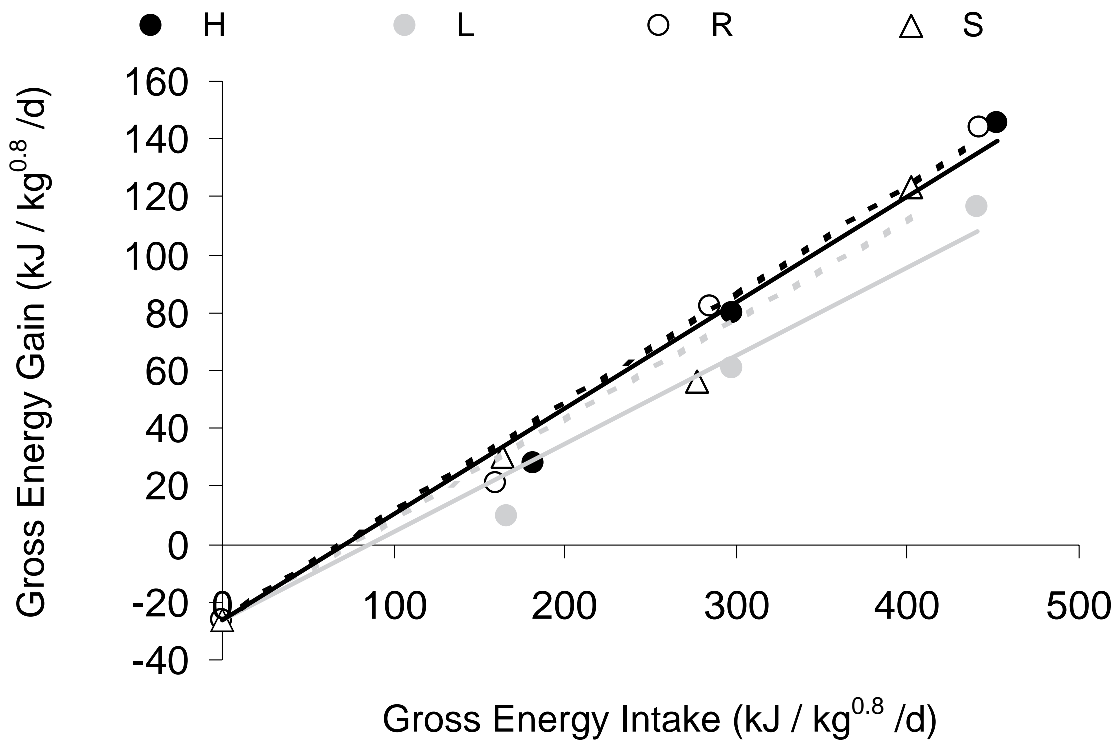

Figure 11.1 Gross energy retention as a function of gross energy intake from fish fed diets in Experiment 3. Each data point is a mean (n=3). Regression equation for H is: y = 0.365x – 26, R2 = 0.988. Regression equation for L is: y = 0.304x – 26, R2 = 0.975.

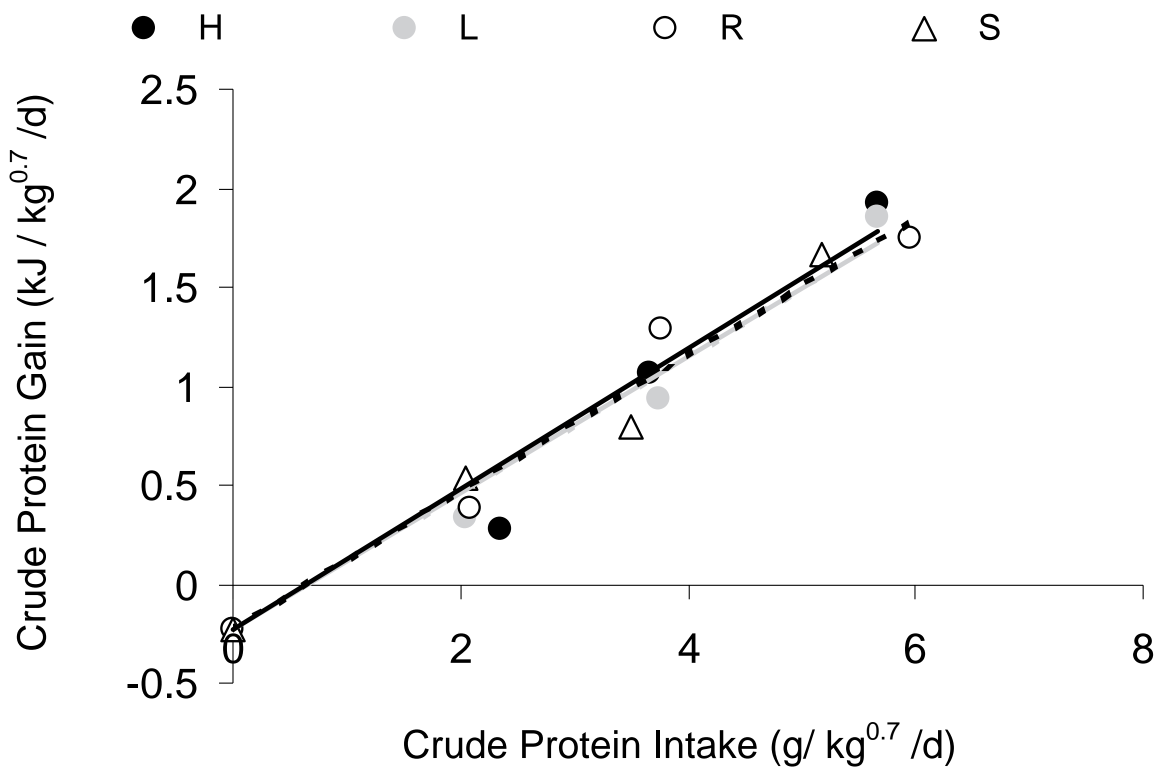

Figure 11.2 Crude protein retention as a function of crude protein intake from fish fed diets in Experiment 3. Each data point is a mean (n=3). Common regression equation is: y = 0.356x – 0.23, R^2 = 0.952.

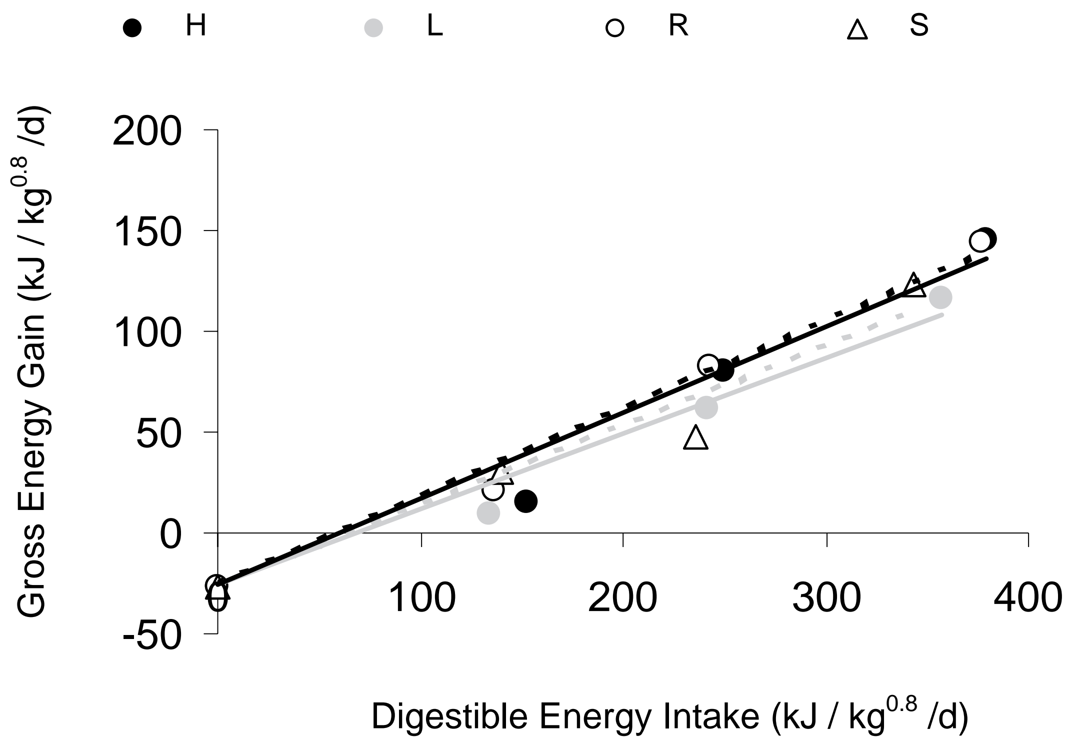

Figure 11.3 Energy retention as a function of digestible energy intake from fish fed the diets in Experiment 3. Each data point is a mean (n=3). Regression equation for H is: y = 0.4264x – 26, R^2 = 0.9613. Regression equation for L is: y = y = 0.3754x – 26, R^2 = 0.9746

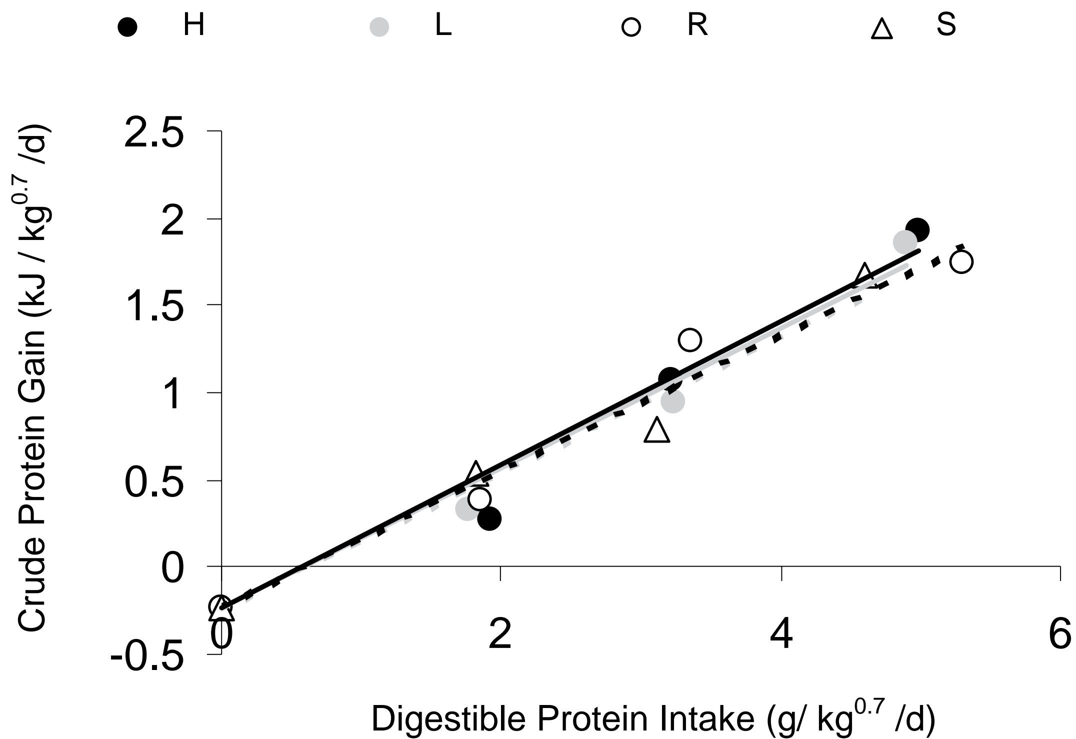

Figure 11.4 Protein retention as a function of digestible protein intake from fish fed the diets in Experiment 3. Each data point is a mean (n=3). Common regression equation is: y = 0.4085x - 0.23, R^2 = 0.965.

12.0 An assessment of different concentration methods on the protein content of lupin products and modelling of theoretical optimal protein concentrate characteristics

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Abstract

A series of practical and theoretical studies were undertaken to examine the potential of increasing the protein content of a variety of grains. A wet-method using ethanol washing and dry-method of particle-size classification were used. Increases in protein were observed using either of the methods. The dry methods were observed to be more effective in increasing the protein content (30% to 41%), but had poor yield efficiencies. The wet extraction methods had lower relative increases in protein (55% to 59%), but had significantly better yields. Modelling of grain protein concentrate use suggested that a product with a protein level in the range of 50% to 60% would be optimal for use in salmonid feeds and provide the most likely economic feasibility and greatest level of replacement of fishmeal.

12.1 Introduction

The need for alternatives to fishmeal as protein resources in aquaculture feeds is well recognised (Naylor et al., 2001). While there is a large range of feed grains suitable for use in terrestrial animal feeds, those feed grains suitable for use in aquaculture feeds are somewhat fewer (Gatlin et al., 2007). For a raw material to figure in any specific formulation it has to not only be cost effective, but also satisfy several risk constraints (e.g. presence of contaminants) and be of a composition amendable to the formulation specifications being sought. These formulation specifications vary depending on the species being fed and the stage of its lifecycle (Glencross, 2006). One of the principal limitations of many feed grains is their inherently low level of protein. The presence of anti-nutritional factors (ANF) in some feed grains also can limit their usefulness as a feed resource (Francis et al., 2001). One way in which both the protein level and ANF issue can be averted is through the production of protein concentrates from grains.

There are several different processing methods that can be used to increase the protein content of grain products. Protein concentration technologies generally use either a "dry" approach or a "wet" approach (Lasztity et al., 2001; Bilgi et al., 2004; Wang et al., 2004; Agren and Ekklund, 2006). A dry approach usually uses a particle size or density differentiation method and has the advantages of not needing to dry the product, which substantially reduces the production cost, however yields are usually poor and the potential increase in protein concentration limited (Reichert, 1982; Cloutt et al., 2006). Wet methods rely on various aspects of protein solubility (or lack of solubility) to enable either the removal of non-protein components to concentrate the remaining protein content, or to solubilise the protein itself and isolate it from the remaining non-protein component. Following either method the product invariably has to be dried and this can affect product quality (Claussen et al., 2007).

There have been a range of methods used in making protein concentrates that have been used in the aquaculture feed sector. Most products have been made using wet methods and base grain products such as soybeans, canola or lupins (Kaushik et al., 1995; Refstie et al., 1998; Glencross et al., 2004a). However, Booth et al., (2001) reported the evaluation of an air-classified lupin protein concentrate. This dry method uses density differentials to separate out protein dense parts of a grain meal from less dense fibre-rich parts of the meal. The key issue to the use of either method are the prospective gains in protein concentration achievable from the base grain and also the potential yield.

This study examines two different methods of protein concentration, using several varieties of lupins as a base material, to examine the potential for the development of protein concentrates from these feed grains. The results are then examined in context with a series of modelling studies on the composition needs of a protein concentrate for the aquaculture feed sector.

12.2 Materials and Methods

12.2.1 Ingredient sources

Seed of *Lupinus angustifolius* (cv. Kalya), *Lupinus luteus* (cv. Wodjil) and *Lupinus albus* (cv. Kiev-mutant) was used in the particle fractionation part of this study. Each of the test grains for the particle fractionation was ground such that they passed through a 2000 µm hammer mill screen to create a coarse seed meal. Kernel meals of *Lupinus angustifolius* (cv. Myallie), *Lupinus luteus* (cv. Wodjil) were obtained from commercial grain processors (Coorow Seed Cleaners, Coorow, WA, Australia). For the ethanol extraction work each of the test meals was thoroughly ground such that they passed through a 600 µm hammer mill screen.

12.2.2 Size fractionation

A 300 g sample of milled lupin seed meal of *Lupinus angustifolius* (cv. Kalya), *Lupinus luteus* (cv. Wodjil) and *Lupinus albus* (cv. Kiev-mutant) was separated into its various fractions using the vibratory sieve. Sieves with an aperture size of 1400 µm, 1000 µm, 710 µm, 500 µm, 212 µm, 125 µm and a collection pan were stacked in descending order. A 300 g sample of each meal was weighed and placed onto the 1400 µm sieve and fixed to a sieve vibrator (Analysette-3 Spartan Pulverisette, Fristsch, Idar-Oberstein, Germany) for 10 min. Following sieving the weights of the sample that have passed into each screen was weighed and their relative amounts determined. A sample was collected from each screen following weighing for subsequent protein analysis.

12.2.3 Ethanol extraction

Samples of kernel meal from either *Lupinus angustifolius* (cv. Myallie) or *Lupinus luteus* (cv. Wodjil) were protein-concentrated using an ethanol solution wash based on the methods of Glencross et al. (2003) and Wang et al. (2004). A 100g sample of either meal was placed in a 250 mL beaker with 200 mL of each of the different concentrations of ethanol (60%, 70%, 80% and 90%), for different periods of time (1, 2, 4, 8, 16, 32 min). Each sample was mixed using a magnetic stirring system. Following each washing period the contents of the beaker were filtered and a sample collected and dried at 90°C for 12h, prior to drying and being analysed for their nitrogen content.

12.2.4 Chemical analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Samples were analysed for dry matter and protein content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25.

12.2.5 Statistical analysis

All values are means unless otherwise specified. Effects of ethanol concentration and washing time on the increase protein content of the products were examined by two-way ANOVA. All statistical tests were conducted using Statitistica v6 software. Surface fitting of the data was undertaken using both Microsoft Excel. Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

12.2.6 Opportunity-cost modelling

A series of formulations were costed for a diet of 450 g/kg protein and 22.5 MJ/kg gross energy (Salmon 2, Table 12.2) using the software Winfeed 2.8 (Cambridge, UK). Only diet protein, starch and energy densities were fixed as formulation parameters, no allowance was made for the impact of any hypothetical GPC on the amino acid composition of the diet. The only other protein source made available in these formulations was a hypothetical grain protein concentrate (GPC). These formulations examined the cost that could be afforded for the hypothetical GPC of varying protein content and 50 g/kg of lipid, the remainder being of non-nutritive value (assumed as 30 g/kg Ash and the remainder as non-starch polysaccharides). The maximum values of the GPC and their respective maximum inclusion levels are indicated in Figure 12.6.

12.3 Results

12.3.1 Fractionation

There was a similar effect of particle-size fractionation on each of the different lupin seed meals (Figure 12.1). The highest protein concentration was found in the finest fraction (< 125 µm), with the lowest protein concentration found in the particle > 500 um. These effects were generally consistent across all three lupin varieties.

From a base protein level of 30%, the *L. angustifolius* fractionation had the greatest increase in protein concentration (122%) in the < 125 um fraction. From a base protein level of 38%, the *L. angustifolius* fractionation had the greatest increase in protein concentration (121%) in the < 125 um fraction. From a base protein level of 36%, the *L. albus* fractionation had its greatest increase in protein concentration (137%) in the < 125 um fraction. However, yields in each of these fractions were nominal (< 1%). The combination of all grain fractions less than 500 um would substantially improve the yields to be 17%, 26% and 35% for *L. angustifolius*, *L. luteus* and *L. albus* respectively. Relative increases in protein for these higher yielded products would be somewhat less at 103%, 111% and 106% for *L. angustifolius*, *L. luteus* and *L. albus* respectively.

12.3.2 Ethanol extraction

There was a significant effect of both ethanol concentration (P=0.000) and washing time (P=0.031), on the increase in protein content of the concentrates made through wet-extraction of *L. angustifolius* kernel meal. A significant interaction effect was also observed between ethanol concentration and washing time (P=0.000). The greatest relative increase in protein content from the base material was observed with the 60% ethanol washed for 32 minutes, with an increase in protein concentration to 43.5% (increase of 104%). Product yields were > 90%.

There was also a significant effect of ethanol concentration (P=0.000) but not washing time (P=0.220), on the increase in protein content of protein concentrates made through wet-extraction of *L. luteus* kernel meal. A significant interaction effect was also observed between ethanol concentration and washing time (P=0.000). The greatest relative increase in protein content from the base material was observed with the 60% ethanol washed for 32 minutes, with an increase in protein concentration to 59.5% (increase of 107%). Product yields were greater than 90%.

12.3.3 Opportunity modelling

A series of models were created to determine the effect of different protein concentrations, of a hypothetical GPC, on the opportunity cost of using such a product in a salmonid feed. In these models the GPC was the sole protein source replacing fish meal in each case. Fish meal price (AUD$1,200 per tonne) and composition (65% protein, 9% fat) were fixed. The formulations were also fixed across each of the models based on a diet of 450 g/kg protein and 22.5 MJ/kg gross energy (diet - Salmon 2, Table 12.2). The price of other key ingredients was; fishoil: $1000, wheat: $240, vitamin premix: $5000.

In this model, the formulations showed that the cost that could be afforded for the hypothetical GPCs increased with increasing protein content of the GPC (Figure 12.6). The maximum inclusion level for a GPC was observed for the 65% protein GPC at 67% inclusion, which allowed complete replacement with the fishmeal content of the diet. Above 55% protein, the maximum opportunity cost for a GPC exceeded AUD$1,000 per tonne. At the lowest protein level examined (45%) an inclusion level of 8.9% was derived, with an opportunity cost of ~AUD$830 per tonne.

12.4 Discussion

The need for an alternative to fish meal as a protein source in aquaculture feeds has been well documented (Naylor et al., 2001; Gatlin et al., 2007). While there are many feed grain options that are widely used in the terrestrial animal feed sector, there is a comparative paucity of feed grain options for use in aquaculture feeds (Gatlin et al., 2007; Glencross et al., 2007). To address this issue and improve the level of risk associated with reliance on fish meal there is a need to examine the options for value-adding grains to produce a protein concentrated product that suits the needs of this feed sector. Ideally, this product will need to be low-cost to be competitive, but there are likely to also be other functional composition constraints on what is needed to serve this feed sector in terms of a protein concentrated product.

12.4.1 Effects of protein concentration on ingredient composition

In an effort to examine the preliminary possibilities of simple protein concentration options a particle classification and wet-extraction process were examined using a variety of lupins as the base material (Riechert, 1982; Wang et al., 2004; Agren and Eklund, 2006; Cloutt et al., 2006).

The size-fractionation study showed that it was possible to use particle size differentials to concentrate protein of all three lupin varieties. In this study seed meal was used at the starting material to minimize the cost of the base material. However, it may be prudent to re-evaluate this work based on the use of kernel meals also.

Other studies using air-stream classification methods have also shown significant capacity to increase the protein content of both field peas and lupins (Riechert, 1985; Evans, 1999). Field peas in particular show good application in particle-classification processes although the starting protein content of the meal, similar to the present study, has been shown to have a significant effect on the protein content of the resultant GPC (Riechert, 1985). Although highest protein concentration was found in the finest fraction (<125 µm) the yield of this fraction for all three lupin varieties was nominal and certainly not worthy of consideration as a useful means of GPC production. However, if all grain fractions less than 500 µm were combined there is a substantial improvement in the yields to 17%, 26% and 35% for *L. angustifolius*, *L. luteus* and *L. albus* respectively. The downside to this increase in yield though is that the relative increases in protein for these higher yielded products would be somewhat less at 103%, 111% and 106% for *L. angustifolius*, *L. luteus* and *L. albus* respectively. These dry-methods should also be re-evaluated with field peas, which post-extrusion may also offer some capacity for co-product development of pea starch as well as a GPC.

The wet-method showed marginal increases in protein content of both lupin varieties. Although the relative protein increase was not as much as that observed fro the dry-method the absolute protein levels and the yields were significantly better. Notably these are two key factors affecting the viability of any GPC produced. It was also noted that the protein content was increasing with increased duration of mixing and also with more dilute ethanol solutions. It may be possible to further optimise these processes by expanding the limits of this study. Heating the ethanol solution may also improve the solubility of any soluble fibres to be removed (Carre et al., 1985; Petterson, 2000).

The wet-method also confers significant opportunities to not only concentrate the protein content of the grain, but also remove or modify any anti-nutritional factors. Glencross et al. (2003) used ethanol washing to remove the oligosaccharides from lupin meal in diets fed to trout. This was found to significantly improve the digestibility of protein and energy in the meal. The negative aspect to the wet method though is that it requires a drying phase and this is likely to draw significant costs into the process. The use of heat in drying grain products has also been shown to affect the functionality of the protein and also affect its nutritional value (Glencross et al, 2004c; Claussen et al., 2007).

A greater degree of comparability could be made between the two methods if the same starting material was used in each case. However, the commercial viability of kernel meals and that these already satisfy many of the modelled GPC requirements supports that there is little value in pursuing this further with seed meals. Future GPC work should focus on kernel meals as a base material.

12.4.2 Modelling optimal protein concentration of a grain protein concentrate for aquaculture feeds

The term “aquaculture feed” is somewhat of a generalisation, as there are numerous types of diets, depending on species and age of the animals being fed (Table 12.2). Typically, modern feeds designed for younger, smaller fish tend to be high protein (> 500 g/kg) and are moderately energy dense (< 20 MJ/kg), while feeds for larger and older fish tend to be lower in protein (400

to 450 g/kg) and are more energy dense (> 21 MJ/kg) (Webster and Lim, 2002). Typically such feeds have a high fat content to maximise the dietary energy intake. These types of feeds are often referred to as high-nutrient-dense (HND) diets.

By contrast there is also a range of diets for species that are either unable to deal with high dietary levels of lipids, or their large gustatory capacity makes it practical to feed them on lower-cost, less energetically dense diets. For example, a prawn diet has a protein level not dissimilar to that of a salmon or barramundi diet, but because they are unable to deal with high dietary lipid levels the total dietary lipid content must be restricted to less than 100 g/kg (Glencross et al., 2002). Abalone diets also have similar limitations (van Barneveld et al., 1998). Tilapia are a species that has a large gustatory capacity and can compensate the use of low protein diets by consuming sufficient amounts of a low-energy dense diet to satisfy its demand for protein for growth. These types of diets are often referred to as low-nutrient-dense diets (LND).

One of the fundamental constraints to HND diets is the limited formulation flexibility that exists. The capacity to use ingredients that do not contribute useful nutritional material is limited in these diets. In contrast, LND diets have considerably more capacity to accommodate ingredients with additional non-useful nutritional content. The capacity that each of the different diets have to accommodate this non-useful nutritional content is estimated in table 2 under the term of "space", with the higher the amount of "space" the greater the capacity to accommodate non-useful nutritional content. This concept of formulation "space" has important implications for the development of any protein concentrate for this sector.

It is recognised that the higher the protein content of an ingredient then the higher it's potential value (Figure 12.5 and 12.6). In addition, protein sources with functional properties are also likely to command premiums. The highest value noted on figure 12.5 is that of wheat gluten that commands this high price because of the high value placed on its functional properties by the food industry. A plant derived protein concentrate for aquaculture feed use though doesn't necessarily have to have specific functional properties, but its use is likely to be highly price sensitive. Accordingly, keeping the cost/price of such an ingredient to an effective level will depend on many things. One important step is the determination of prospective protein levels at which the ingredient is likely to be cost-effective to both produce and use. This issue becomes further complicated by the fact there are two key strategies that can be used to increase the use of alternative ingredients. One uses the basis of sole substitution and the other, dual substitution, requires the complimentary use of an accessory low-value ingredient.

In this hypothetical scenario optimising the protein level (and by default the non-useful content) is the key to defining the most useful product. The determination of an "ideal" protein level can be determined using a variety of methods and is also likely to be somewhat formulation dependent. It is also likely to be dependent on the cost and composition of other competitive ingredients in the feed market. Accordingly these optimal composition and values are only estimates and would be better evaluated under a broader range of assumptions, including the options of other competitive ingredients.

Although a somewhat simplistic evaluation, least-cost linear formulation with hypothetical ingredients can show the relationship between diet formulation, ingredient composition, potential ingredient value and likely inclusion level (Figure 12.6). The limitations of this evaluation are that the inclusion levels and price of the hypothetical ingredients are highly dependent on the price and composition of fishmeal. What this approach does define is that the "ideal" protein level is from 500 g/kg to 600 g/kg (Figure 12.6). Over this protein range the GPC is included in the diets at between 11% to 26%. Above this protein range (50% to 60%), the complete

replacement of the fishmeal occurs and risk is merely transferred from fishmeal to the GPC and the overall formulation risk is not reduced at all (Glencross et al., 2007).

Ironically several raw materials already exist that fit within this spectrum, notably kernel meals of *L. luteus* (Glencross et al., 2004b), but also feed grade corn gluten and wheat gluten products are also available that cover a similar nutrient profile (Table 12.1). Several soybean protein concentrates that have these specifications have also been tested (Refstie et al., 1998). Many rendered animal meals (bovine, ovine and poultry) also have protein specifications within this range (Table 12.1).

Beyond this simplistic scenario, the issue becomes predominantly a price sensitive one and competition among other ingredients reduces the effective price of some ingredients. Notably, the hypothetical maximum price for a GPC of 50% protein was $913, where as soybean meal at 49% protein (as-fed basis) is worth only $450 per tonne. Notably, while the modelling results show a linear value of the GPC with increasing protein content (Figure 12.6), actual values of ingredients against their protein content show that this is more likely an exponential relationship (Figure 12.5). Further modelling using actual price and composition data of existing feed ingredients would increase the robustness of this assessment and provide a more realistic value determination model. In addition, modelling using a variety of diet specifications would also provide a broader assessment of the likely specifications required for a range of diets.

An improved way to assess the optimal protein level for a hypothetical protein concentrate would be to use non-parametric modelling. In this scenario the assumption parameters for the model are not fixed *a priori* and therefore the modelling approach maximises its flexibility in being able to identify possible outcomes to service a range of needs. This approach is used in some manufacturing industries to define certain product parameters (Gani, 2004).

12.4.3 Conclusions

The findings of this study show that both dry and wet methods can be used to produce a value-added grain protein product. The dry methods were observed to be more effective in increasing the protein content, but have very poor yield efficiencies. The wet extraction methods had lower increases in protein, but had significantly better yields. It is difficult to directly compare both methods directly in this study as the base materials were different in each study. Further work examining the potential of varying the wet extraction methods would be worthwhile, as would a direct comparison of size- or air-classification of lupin kernel meals with those wet extraction methods.

Modelling of GPC use suggests that a product with a protein level in the range of 50% to 60% would be optimal for use in salmonid feeds. Further assessment of the “ideal” product specifications needs to be undertaken with a broader range of diets as the “ideal” product specifications are likely to vary depending on the diet in which they are being applied to.

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Tables and Figures

Table 12.1 Composition of ingredients. Details are on a dry matter basis (g/kg DM) unless otherwise specified.

Ingredients	AKM	LKM	SBM	PEA	CAN	WGL	CGL	POU	FSM
Dry Matter (g/kg)	885	903	909	903	920	910	920	920	920
Protein	415	547	518	257	394	838	600	600	718
Fat	53	87	47	12	82	9	25	120	105
Carbohydrate	499	321	365	703	460	146	278	0	0
Ash	33	44	69	28	65	8	20	200	152
Organic Matter	967	956	931	972	935	992	900	720	848
Phosphorus	4	6	8	5	11	2	4	17	26
Energy (MJ/kg DM)	20.4	20.9	19.6	18.6	20.5	22.6	19.9	18.8	21.5
Typical price ($/tonne)	350	500	450	300	300	3000	1000	800	1200
Price ($) / g Protein	0.84	0.91	0.87	1.17	0.76	3.58	1.66	1.33	1.67

Typical prices are approximate based on a USD : AUD exchange rate of 1 : 0.75 and cif Australia. LKM: *L. luteus* kernel meal; AKM: *L. angustifolius* kernel meal; SBM: Solvent-extracted soy bean meal; PEA: Field pea (*Pisum sativum*) meal; CAN: Solvent-extracted canola meal; WGL: Wheat gluten; CGL: Corn gluten; POU: Poultry meal; FSM: Chilean Prime Anchovy meal. Data derived from unpublished data (B. Glencross).

Table 12.2 Generalised composition (g/kg as-fed) of diets for various species, including an indication of the typical amount of formulation "space" available.

	Salmon 1	Salmon 2	Salmon 3	Barra 1	Barra 2	Prawns	Tilapia	Abalone	Marron
Dry Matter	920	920	920	920	920	920	920	920	920
Protein	400	450	550	450	500	450	300	300	250
Fat	300	250	200	200	130	80	80	50	50
Starch (min)	100	70	70	70	70	100	200	70	70
Other "essentials"	50	50	50	50	50	150	50	50	0
Energy (MJ/kg)	24.0	22.5	22.0	21.5	20.5	19.0	18.0	17.5	17.0
"Space"	70	100	50	150	170	140	290	450	500

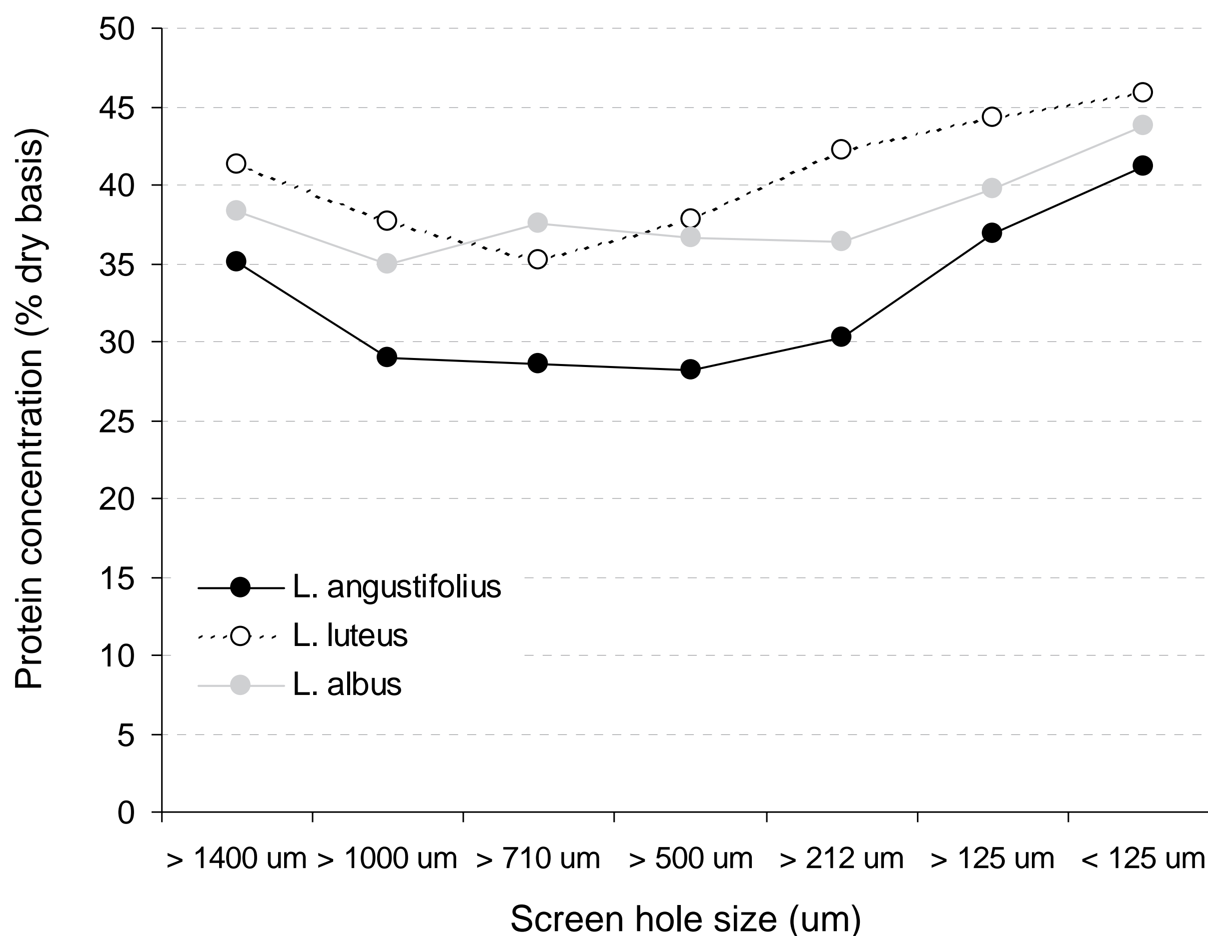

Figure 12.1 Influence of lupin variety and particle size class on the protein content of each size class following screening of coarse-milled seed samples.

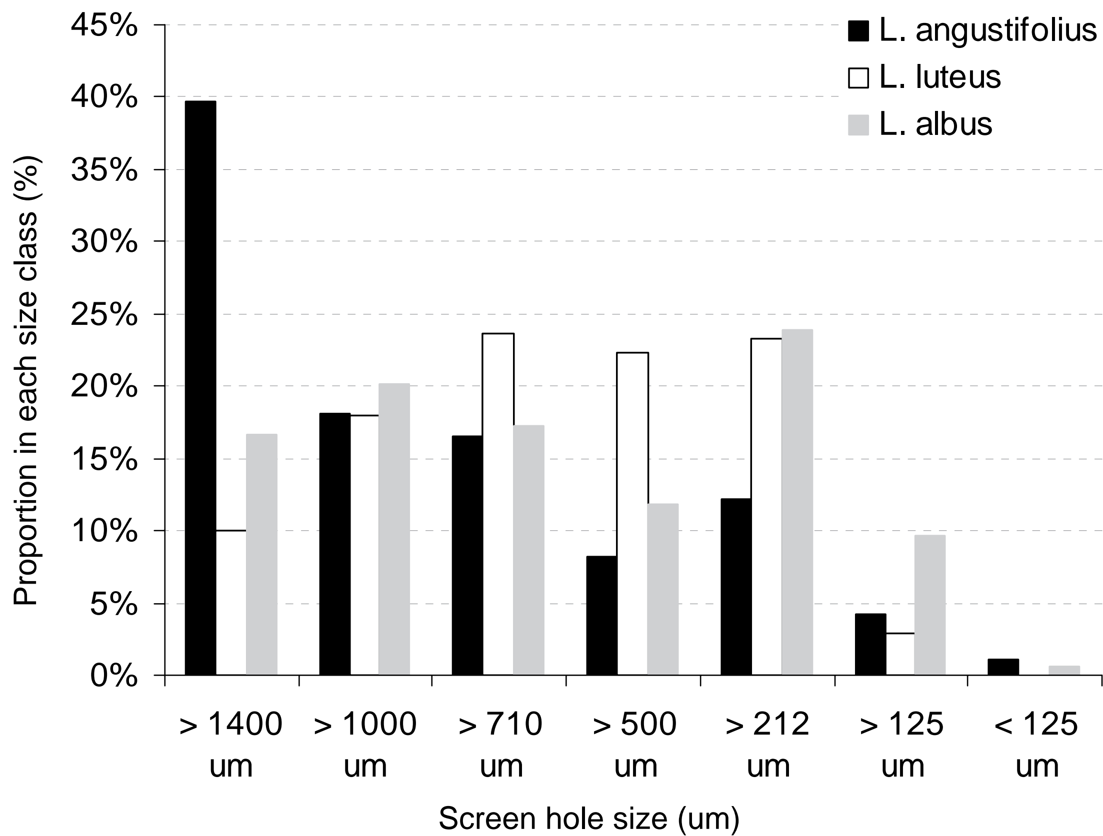

Figure 12.2 Influence of lupin variety on the proportions of a coarse-milled sample present in each particle size class.

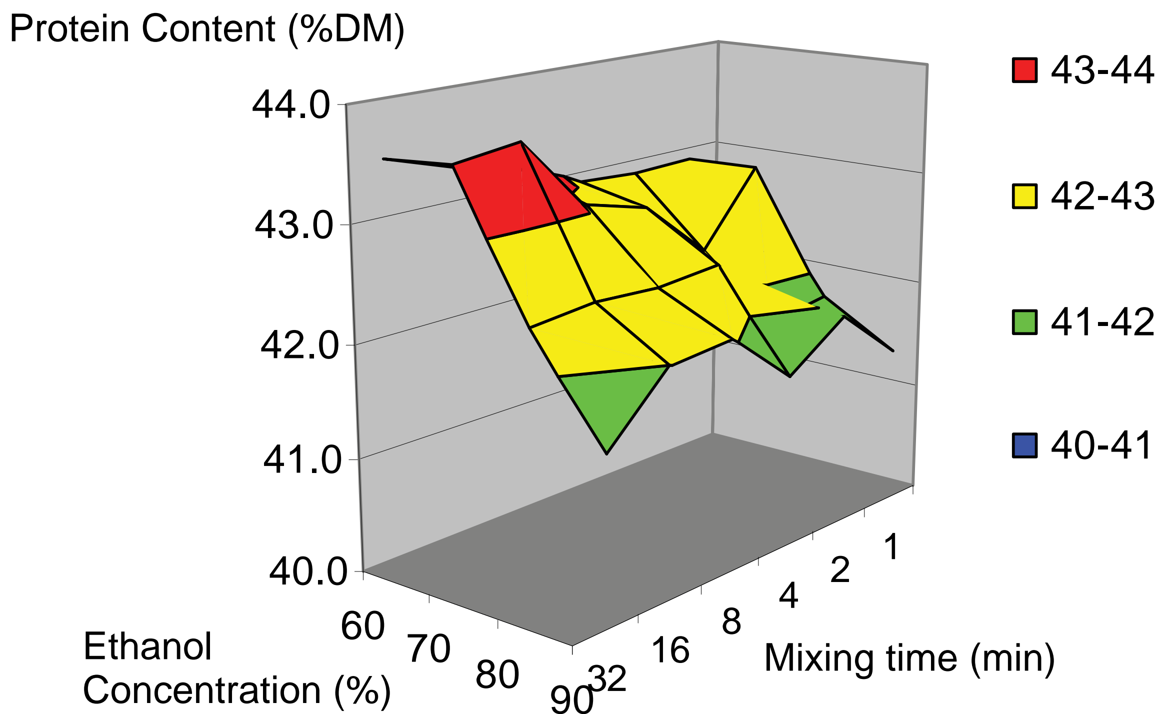

Figure 12.3 Effect of ethanol concentration and mixing time on the protein content of the concentrated *L. angustifolius* product. Protein content of the initial *L. angustifolius* kernel meal was 41.7%DM.

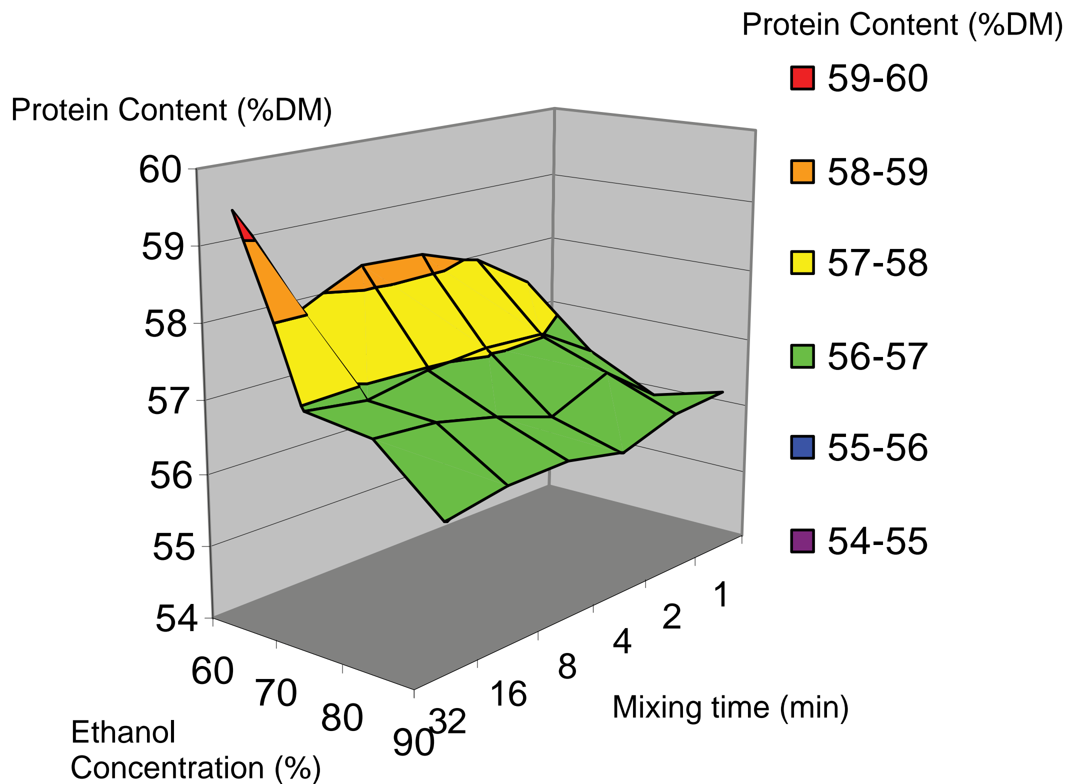

Figure 12.4 Effect of ethanol concentration and mixing time on the protein content of the concentrated *L. luteus* product. Protein content of the initial *L. luteus* kernel meal was 55.5%.

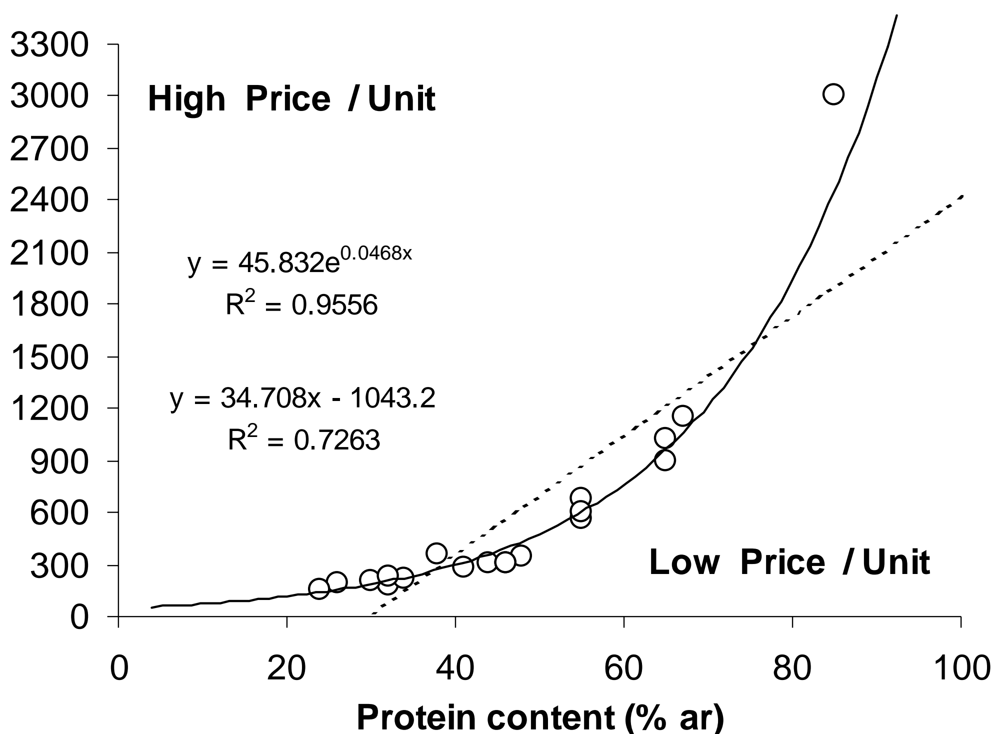

Figure 12.5 Price (AUD$/t) of various feed grains and grain products based on 2002 data with all product costs on an f.o.b. basis. Shown are two regression options; linear and exponential functions. Points below the line represent products of low price per unit, while those above the line represent products of high price per unit.

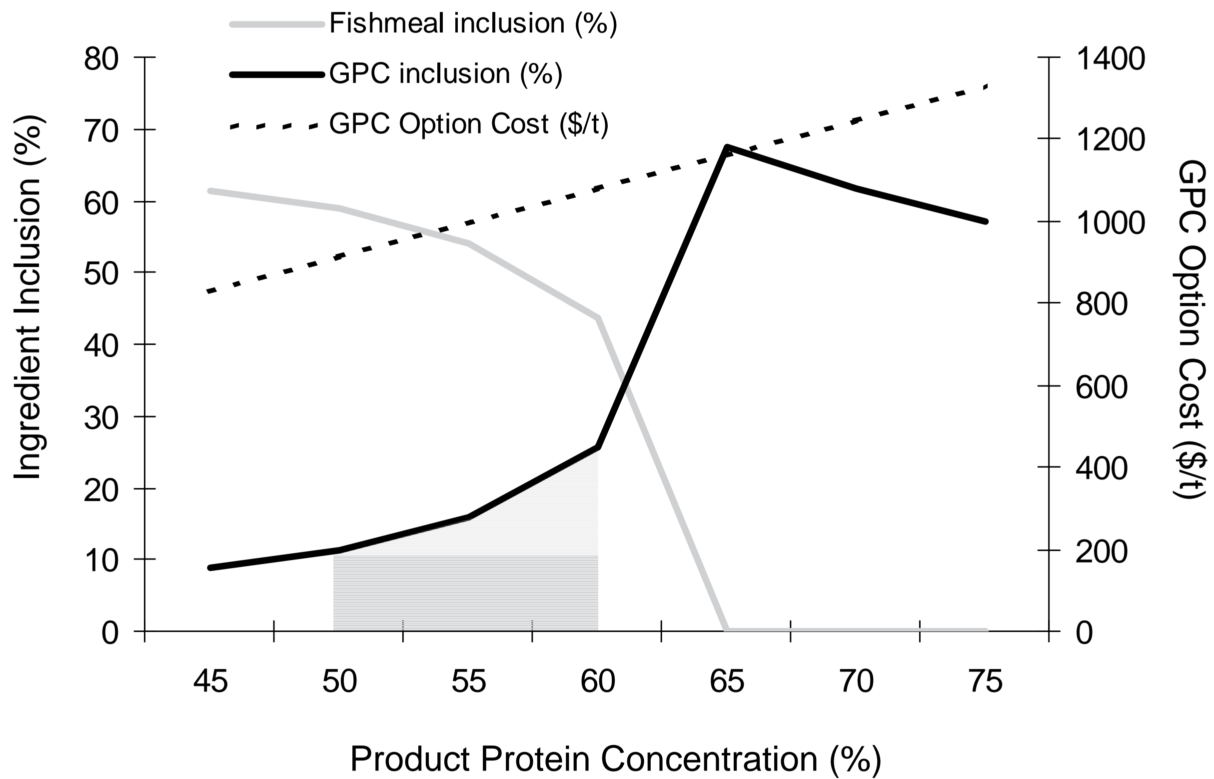

Figure 12.6 Influence of protein level of hypothetical protein concentrates on inclusion level and ingredient value ($/tonne) when included in a single type of HND diet (450 g protein/kg and 22.5 MJ). Shown in shading are the ranges of protein level that would be optimal for production of a grain based protein concentrate for this diet specification.

13.0 Development of protein concentrated lupin products for use in aquaculture feeds

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Abstract

Protein concentrates and isolates were made from lupin kernel flours using standard soybean industry processes. *Lupinus angustifolius* kernel flour with starting protein content of 46.1% crude protein (CP) (N x 6.25) achieved a protein content of 52% CP after washing with a 70% ethanol solution (10:1) while the *L. luteus* lupin kernel flour with starting protein content of 52.0% CP achieved a protein content of 71.7% CP. The fraction of the flour which is removed by washing is predominantly oligosaccharides which make up 8% of the *L. angustifolius* kernel flour and 14% of the *L. luteus* kernel flour. *L. luteus* flour reached the 65% benchmark set by the soy industry for protein concentrates. Pre-heat treatment of the lupin kernel flours makes the lupin proteins less soluble in the aqueous medium allowing for the oligosaccharides to be removed with water without the need for ethanol. Lupin protein isolates were prepared from *L. angustifolius*, *L. albus*, *L. luteus* and *L. mutabilis*) by solubilisation at pH 9, removing the insoluble residue (fibre) and acid precipitation (pH 4.5). Overall the protein recoveries are similar (~ 85% to 90%) for all four species with the exception of *L. albus*, which was approximately 10 per cent lower. *L. luteus* and *L. mutabilis* appear to be excellent protein sources for the production of protein isolates given the high initial kernel protein concentrations. However, a de-fatting step would have to be introduced for *L. mutabilis*. Particle size of the kernel flour influenced the amount of protein lost with the fibre fraction. Extended soaking of the kernel flour in water at native pH5.5 resulted in significant protein recoveries. Pectinase treatment did not improve the protein extraction yields. Given the yields and production costs, concentrates from *L. luteus* appear to be the most viable option for production of a grain protein concentrate for use in the aquaculture feed industry.

13.1 Introduction

Global fishmeal production from wild-catch sources cannot continue to increase indefinitely, suitable alternatives have to be found for sustainable aquaculture. The growing need for protein (food & animal feed including fish) and of protein-enriched products has resulted in an intensive search for new protein sources. Plant based aqua feeds seem to be the ideal alternative but have their own limitations, primarily their lower crude protein content relative to fishmeal and the suite of anti-nutritional factors that accompany them. Plant based ingredients typically are too dilute in their protein content to replace fishmeal 'one for one' which creates 'space' issues within high specification diets that are used for instance in the salmon industry (Glencross, 2003; Williams, 2007). Accordingly the preparation of 'protein concentrates', from plant sources would seem to address the issues of increasing the protein and eliminating the anti-nutritional factors (Glencross et al., 2003). To date the major plant protein used in the food and feed sectors is soybean due to its high protein content, good nutritional value and lower price compared with animal proteins. Soybeans were the first plant protein source for the production of protein concentrates and isolates. Initially these protein products were used by the food

ingredient markets (Lusas, 2004) however, more recently these refined ingredients are being utilised by niche markets in the feed sector (Dersjant-Li and Peisker, 2004).

Given the comparative similarity of lupins to soy there has been considerable interest in evaluating the potential for value-added products, such as lupin protein concentrates and isolates. Based on a series of theoretical planning exercises and modeling studies the optimal composition of a value-added protein product for the aquaculture feeds industry was identified to be between 50% and 65% CP, price contingent (Glencross, 2003; Sipsas, 2003).

13.1.1 Lupins as starting material for concentrates and isolates

There are several species in the genus *Lupinus*. The economically significant species include *L. albus* (albus) the 'European lupin', *L. luteus* (yellow lupin or YL) mainly grown in Germany and Eastern Europe, and *L. angustifolius* (narrow leafed lupin or NLL), the main lupin grown in Australia and in particular Western Australia (Petterson, 2000). Recent investigations into the potential of *L. mutabilis* (mutabilis) in the West Australian cropping system are looking promising and will continue (Sweetingham et al., 2006), given this lupin species is the most suited to processing for value-added products (Aguilera, 1988).

The gross chemical composition of these four lupin species are shown in Table 13.1. Both the whole seed values as well as the dehulled kernels (cotyledons) are reported. Dehulling is the first step in the process of producing a 'protein enriched lupin product'.The most significant values are the kernel protein levels of both YL and mutabilis. However it needs to be noted that the seed coat accounts for 27% of the YL seed and 16% of the mutabilis seed, therefore impacting on dehulling yield quite significantly with 73% and 84%, respectively.

13.1.2 Objectives of this study

The objectives of the study presented in this chapter include:

- The examination of standard concentrate processing technologies on the protein yield of products produced from lupins.
- The examination of standard isolate processing technologies on the protein yield of products produced from lupins.
- The effect of lupin species on the protein yield of different products.

Protein concentrates are considered to have greater than 50% protein and are essentially flour (dehulled kernels) from which the carbohydrates (free sugars and oligosaccharides) and other soluble materials have been removed (Lusas, 2004) by an aqueous wash.

Protein isolates are defined as the major protein fraction of soybean prepared by removing most of the non-protein components. The technology associated with protein isolates is well known (Lusas, 2004). The protein is extracted with water at alkaline pH to yield a soluble protein and a protein exhausted residue (fibre). The fibre is removed, and the soluble protein is then precipitated at pH 4.5-5.0 to yield a protein curd and a legume whey. The curd is then washed (may be neutralized) and dried (usually spray dried) and is then called an isolate. They contain not less than 90% protein on dry basis. This definition was approved for soy by the United States Food and Drug Athority in 1961 (Manrique, 1977) and is commonly accepted for other legumes for the food industry.

Although these are the benchmark levels required for a protein isolate or concentrate in the

soybean industry the levels may vary between 50%-90% protein depending on end use. In some instances when the isolate products do not meet the 90% CP (dry basis) they are called protein concentrates even though they were made by the isolate method. For example the dairy industry have developed protein concentrates and isolates from milk using an isolate method. The level of protein in the final product determining the definition of the product.

13.2 Methods

13.2.1 Concentration process

13.2.1.1 Raw materials

Whole NLL cv. Mandelup and YL cv. Wodjil seeds from Wongan Hills (Western Australia) grown during the 2003 season, were dehulled using a SKV- dehuller. The kernels were milled to pass through a 250μm screen. The kernel flour was either left raw (without preheat treatment) or preheated (autoclaved) at 122°C for 1hr (inclusive of ramp up, ramp down and depressurisation).

13.2.1.2 Standard concentration method

A 30g sample of raw kernel flour was mixed with 300mL of 70% ethanol and stirred for 1 hour at 25°C by centrifugation (5 mins at 8000 rpm at 20°C). The supernatant was poured off and the residue dried. The dry weight of the residues (concentrates) was recorded and the CP, Fat, Ash, Lignin, Oligosaccharides and Kernel polysaccharides (dietary fibre) were analysed.

13.2.1.3 Optimisation of the concentration method

Protein concentrates are commercially produced by various processes including:

- by denaturing the flour with moist heat (preheating) and then washing with water.
- separation of the sugar fractions (oligosaccharides) by extracting with an ethanol solution.
- addition of heat during the aqueous stage.
- washing away the carbohydrates at the isoelectric point (based on the fact that the major part of the native protein is insoluble in acidic (pH 4.5–5.0) aqueous solutions.

To optimize the concentration process a number of treatments were trialled using the two lupin kernel flours (Mandelup and Wodjil). The treatments included the washing of all flours at 65°C and 25°C using a range of ethanol concentrations (0-60%).

A 20g sample of kernel flour (raw or heat pre-treated) was mixed with 200mL of either distilled water or a range of ethanol solutions (20%, 40% 60%) stirred for 1 hour (25°C or 65°C) then decanted into a 500mL centrifuge tube with additional water to make up to 400mL and centrifuged (5 min at 8000 rpm at 20°C). The supernatant was poured off and the residue dried. The dry weight of the residues (concentrates) was recorded and the CP was analysed using the Leco FP-2000.

13.2.2 Isolation process

13.2.2.1 Raw materials

Lupin (*L. angustifolius* cv. Myallie) seed was obtained from Wongan Hills 2001(Western Australia). The lupins were dehulled (SKV- dehuller) and milled (Retsch ZM200) to produce three grades of kernel meal:

- Kernel flour: Milled to pass through a 250 μ screen.
- Kernel fine meal: Milled to pass through a 500 μ screen.
- Kernel coarse meal: Milled to pass through a 1000 μ screen.

Albus, YL, and Mutabilis seed was obtained from Wongan Hills 2001(Western Australia). The lupins were dehulled (SKV- dehuller) and milled (Retsch ZM200) to coarse kernel meal (1000μ screen).

13.2.2.2 Standard protein isolate extraction method

Approximately 30g of lupin flour (meal) was mixed with 300mL of distilled water. 1M NaOH was pipetted drop wise into the sample until pH 9 was reached and maintained by adding 1M NaOH as required for 1 hour. After mixing, the sample was decanted into a 500mL-polycarbonate tube and centrifuged at 8500 rpm at 25°C for 15 min.

The supernatant was decanted into a clean beaker and water was added to the pellet in the polycarbonate tube and again centrifuged. The supernatants were pooled and the pH was reduced to 4.5 with1M HCL and then mixed for 20 min. The pellet or fibre fraction was left in the polycarbonate tube and stored at 4°C.

After mixing the pooled supernatant was decanted into a clean polycarbonate tube and centrifuged once only as described previously. The supernatant was decanted into a clean beaker before adding 5g/100mL of TCA to the solution. The new solution was then stirred for 20 min. The pellet or Protein Isolate1 was left in the polycarbonate tube and stored at 4°C.

After 20 min the sample was decanted into a polypropylene tube and centrifuged once only as previously described. The TCA was then replaced with methanol and shaken intermittently for 15 min by hand and then centrifuged for 10 min. The supernatant was discarded and the pellet or Protein Isolate 2 along with Protein Isolate1 and the Fibre fraction were freeze dried for approximately 12, 24 and 72 hrs respectively. Once dried, the extracts were weighed then milled using a Wiley Mill. The extracts were stored at 4°C.

Protein analysis was determined on a dry basis by the Dumas combustion method using a Leco FP2000 instrument (Leco Corporation, Michigan, USA) after milling the samples on a Retsch mill with a 1.0mm screen (Retsch Co., Germany).

13.2.2.3 Optimisation of the isolation method

To optimise the extraction process a number of treatments were trialed Using NLL cv. Myallie (Wongan Hills 2001). The treatments were arranged into three groups A, B and C.

The groupings were according to the particle size of the starting material as described in *13.2.2.1*.

Group A: The kernels were milled to pass through a 1000 μm screen

Group B: The kernels were milled to pass through a 500 μm screen

Group C: The kernels were milled to pass through a 1000μm (C1) 500μm (C2) and 250 μm screen (C3)

The treatments were as follows:

A1 as for the control (11.2.2.2) except the flour was acid-washed (1h, pH 4.5 HCl).

A2 as for the control, except the flour was slurried in water and allowed to soak overnight.

A3 as for the control, except the flour was slurried in water and frozen (-20°C) overnight (16 hrs.)

A4 as for the control, except the flour was slurried in water and heated to 70°C then allowed to cool.

A5 as for the control, except the alkaline extraction step was taken to pH12 instead of pH 9.

B1 as for the control, except the flour was slurried with pectinase in acetate buffer for 2 hrs.

B2 as for B1, except the flour slurried with pectinase for 16 hrs.

B3 as for B1, except minus the pectinase.

B4 as for the control, except the alkaline extraction at pH 9 was over 16 hrs instead of 1 hr.

C1 as for the control, particle size of flour coarse (5.3% < 300μm).

C2 as for the control, particle size of flour medium (22.2% < 300μm).

C3 as for the control, particle size of flour fine (94.5% < 300μm).

13.3 Results

13.3.1 Lupin concentrates; effect of species and conditions on concentration capacity

The process utilised to produce the lupin (NLL and YL) concentrates mirrors the more common commercial process employed to produce soy protein concentrates (Figure 13.1). By washing the flour with ethanol (~70%) the residue attains an elevated protein level. In NLL approximately 23% of the starting weight, consisting of oligosaccharides, some protein and some fat was eliminated (Table 13.2). In YL approximately 29 % of the starting weight, consisting predominantly of oligosaccharides, some protein and some fat was eliminated (Table 13.3).

The results of the optimisation trial displayed similar trends as for the standard concentration process (Table 13.4). Again YL achieved the higher end protein level and the most amount of 'concentrating' of the protein fraction compared to NLL. The most effective conditions appear to be the pre-heating treatment followed by a straight water wash to achieve the best yields, end protein levels and protein recoveries for both species.

13.3.2 Optimisation of protein isolate extraction efficiency

Approximately 75 per cent of the original weight was recovered as Fibre, Lupin Isolate 1 and Lupin Isolate 2. Lupin Isolates 1 and 2 accounted for 36 per cent of the original weight and 87 per cent of the original protein was recovered in all three fractions (Table 13.5). The basic compositions of these three fractions are:

- Fibre: pectin like polysaccharides made up of galactouronic acid and rhamnose chains (Cheetam, et al., 1993; Evans, 1994).
- Protein Isolate 1: globulin proteins α and β conglutins (Sipsas, 2005).
- Protein Isolate 2: albumin proteins and δ conglutins. (Sipsas, 2005).

The degree of protein exhaustion of the Fibre residue is a key point in driving the efficiency of

the extraction process. The optimum scenario would be to separate the protein from the fibre as cleanly as possible. However a 100 percent separation is not possible as there are proteins chemically bound within the Fibre fraction. To optimise the extraction process a number of treatments were trialled (Table 13.6). Treatments in group-A used a coarse Myallie kernel meal as a starting material. Treatments in group-B used a finer Myallie kernel meal and treatments in group-C analysed the effect of kernel meal particle size.

Treatments in group-A investigated different methods of releasing proteins from the Fibre without using (costly) mechanical energy to produce flour. A coarse meal starting material was utilised and various 'wet softening' methods were trialled including an acid-prewash (A1), overnight soaking (A2), freezing (A3), pre-heating (A4) and overnight soaking with elevated alkaline extractions (A5). As shown, the preheating (A4) and overnight extraction at pH 12 (A5) had no effect. The treatments involving prolonged soaking (including the freezing treatment) of the flour in water did produce fibre fractions with lowered protein levels but these were not converted to increased yields in the protein isolates. Notably both these treatments resulted in significant yield losses and lowered protein recovery. This suggests that at pH 5 (the pH of lupin flour in water) there is a native protease which is active and it appears that the protein was degraded as the yield of Isolate 1 was reduced. Hence the total protein recovery was also reduced. This protease must be inactivated by higher pH values as this degradation was not evident in the overnight extraction at pH 9 (B4) (Table 13.6).

As it seemed that the 'wet softening' approach was not creating significant advances, it was accepted that a finer particle size was a crucial variable in the process. Treatment group-B utilised a 'fine meal' and investigated the use of pectinase as a means of disrupting the polysaccharide chains in the Fibre adequately to release the protein bodies. It also included an extended (16 hrs) extraction at pH 9. As particle size affects kinetics, this treatment would clearly indicate if the 'fine meal' should be finer still. Both pectinase treatments only slightly lowered the residual protein left in the fibre fractions but both significantly lowered the yield of protein Isolate 1 and significantly increased the yield of Isolate 2. The yield and protein recovery losses resulted in both treatments, with the 16 h pectinase treatment the most affected. Both pectinase treatments were conducted in acetate buffer and the buffer (minus pectinase) treatment presented unexpected results (B3, Table 11.6). The acetate buffer affects the extraction significantly, as the Fibre protein is lowered, the protein yield of Isolate 1 is lowered (although not as much as the pectinase treatments) and the protein yield of Isolate 2 is increased by ~ 150 per cent. However there is a total yield loss and lowered protein recovery.

Treatment group-C investigated the effects of kernel meal particle size, which is a critical variable in the efficiency of extracting maximum protein away from the fibre residue as indicated by the results. Fibre protein levels decreased as the particle size of the starting material decreases from 30.8 per cent to 14.4 per cent. Concomitantly there was an increase in yield of the protein Isolate fractions (1 plus 2) from 24.3 per cent to 38.3 per cent as protein is released from the fibre (Table 13.6).

13.3.3 Species effect on isolates

The extraction efficiency for NLL, Albus, YL, and Mutabilis is reported in Table 13.7. Overall the protein recoveries are similar (~85% to 90%) for all species with the exception of Albus, which is about 10 per cent lower. YL and Mutabilis would be excellent protein sources for the production of Protein Isolates given the high kernel protein however a de-fatting step would have to be introduced for Mutabilis.

13.4 Discussion

YL cv. Wodjil achieves a final protein level just under 70% CP and a concentration (increase) of approximately 16% in protein (Table 13.4, Figure 13.2). Contrastingly NLL only achieved a final protein level around the 52% mark with a concentration (increase) of 6-8%. Noticeably there is almost a 15% difference in end protein concentrations between the two flours at the maximum levels achieved. It is interesting to note that the difference in protein of whole grain NLL (30%) vs. YL (38%) is 8% however when subjected to concentration regime the difference widens to 15%.

An important point to note is that the soy industry has set the benchmark at 65 % protein (dry basis) for a product to be classified as a Protein Concentrate. According to the results presented (Table 13.2 & 13.3) only Wodjil reached the minimum protein concentration required.

The yields between the preheated and unheated Wodjil flour for the water (0 % ethanol) treatment showed a 7 per cent increase in yield for the preheated treated flour (Table 13.4, Figure 13.3). This may be due to higher protein solubility of the unheated flour resulting in protein being washed out, whereas the proteins in the heat-treated flour would be denatured and presumably less soluble and retained in the concentrate; resulting in higher yields. However the decrease in yield and protein recovery of the preheated flour, at the 20% ethanol was unexpected (Table 13.4, Figures 13.3 and 13.4).

This was possibly due to an increased solubility of a particular protein in Wodjil under those conditions. Curiously the same pattern was observed with the NLL preheated flour at 20 per cent ethanol. With increasing ethanol concentration the proteins are becoming increasing less soluble leading to a greater yield.

The protein recovery between the preheated and unheated flour for water (0 % ethanol) treatment showed a reduction in protein recovery of approximately 11 per cent for unheated treatment (Table 13.4, Figure 13.4). This may be attributed to high protein solubility of the unheated flour resulting in protein being washed out from the two lupin kernel flours.

13.4.1 Lupin protein isolates; effect of lupin species and processing conditions on extraction efficiency

Protein isolation processes for legumes generally involve a step of protein extraction at alkaline pH where the protein is made soluble. The soluble protein is then precipitated as a protein curd, purified by washing and dried to form a protein isolate. In developing and applying a process for lupins, the commercial soy process was used as a model. Most isolated soy protein products are produced by slurrying flakes/flour with water, then using an alkaline extraction (pH 9), separating the insoluble material from the water soluble protein then precipitating the protein (Isolate 1) with acid (pH 4.5) (Lusas, 2004). When this process is applied to lupin there is a component of the lupin protein that is still soluble at pH 4.5 which needs ultra-filtration for collection (Isolate 2). Isolate 2 has unique food functional properties (Holley et al., 2001) and would represent a significant protein loss (as well as creating a waste problem) if discarded. In the case of lupin the insoluble material, the Fibre is a valuable by-product, which also has useful functional properties (Evans and Htoon, 1996) and health benefits (Archer, et al., 2004; Johnson, et al., 2003 and Hall, et al., 2005).

13.4.2 Standard extraction conditions

Several variables influence the ability to disperse protein including the pH of extraction, particle size of the kernel meal, temperature, duration of extraction, water/meal ratios and ionic influences. Manrique (1977) investigated these factors comprehensively for NLL cv. Uniwhite. As the main objective was to investigate the effect of variety on extraction efficiency the extraction process was designed emulating Manrique's findings. The extraction procedure followed the scheme shown in Figure 13.5. As evident from this scheme there are 3 distinct fractions which are isolated by this process; Lupin; Fibre, Protein Isolate 1 and Protein Isolate 2.

13.4.3 Drying

Drying the 'washed flour' after the concentrate method and drying the 'washed protein curd' after the isolate process, has been identified as the single biggest cost factor in the production of lupin protein concentrates and isolates.

Spray drying of lupin protein isolates has been investigated and shown to work quite effectively by others (Manrique, 1977, Holley, 2001). Spray drying involves transforming a fluid into a dry-powdered form. This is achieved by atomising the fluid into a drying chamber, where the liquid droplets are passed through a hot-air stream. The objective is to produce a spray of high surface-to-mass ratio droplets, then to uniformly and quickly evaporate the water. Evaporation keeps product temperature to a minimum to prevent high-temperature deterioration. Spray drying is used for products as diverse as chemical, pharmaceuticals and food products such as skimmed milk.

A key factor is the Total Solids Content (TSC) of the final protein curd, which can be introduced to the spray dryer. Typically the most which can be achieved before the viscosity becomes limiting with lupin protein curd is around the 24-27% TSC, which requires that for every one kilogram of dried protein recovered approximately 4 litres of water need to be driven off. Given the current pricing structures (2005-2006) in Australia drying costs can be expected to be between $0.40 -$0.80 per kilogram ($AUD 400-800/tonne) (Saurin Group of Companies, 2003).

The area which has not been investigated, is a suitable drying technology and associated costs of drying the 'washed flour' from the concentrate method. Given the very particulate form of the 'washed flour' (concentrate process) compared with the more fluid form of the protein curd (isolate process), it would be expected that a different drying technology would be needed for this system.

The most appropriate drying technologies to employ would be either the ring dryer or the fluid bed process. The ring dryer has been used to dry products in many industries including food, chemical, mineral and plastics. A broad range of feed materials including powders, cakes, granules, flakes, pastes, gels, and slurries can be processed. Alternatively the fluid bed process requires that the solids are in particulate form prior to entering the fluid bed.

13.4.4 Potential for Industrial Scale up

YL makes an excellent starting material for the production of protein concentrates, in many instances far exceeding the 65% benchmark set by the soy industry. However NLL are not a good starting material for protein concentrates as the modest 6-8% increase in protein achieved does not warrant the expense of the process. NLL protein concentrate levels of 52-53% leaves it well below the benchmark set by the soy industry. It is relevant to note that dehulled YL kernel

meals have a 50-52% CP level without the need for wet processing. The key issue for yellow lupin concentrate becoming a competitive product in aquaculture, given its excellent nutritional and functional properties (Glencross et al., 2005) is the cost-effectiveness of the drying process. This has yet to be ascertained.

In terms of producing isolates both YL and NLL performed equally. Lupin isolates, both fibre fractions and proteins have potential in the high value food ingredients sector (Sipsas, 2005), however the yield and cost of producing lupin protein isolates would make them prohibitively expensive for the aquaculture industry, except for 'niche' market applications.

13.4.5 Conclusion

There has been considerable interest in the potential for value-added products, such as lupin protein concentrates and isolates, though it has to be noted that the aquaculture feeds sector is only likely to regard such products solely on a price per unit value basis.

Accordingly initiatives in the development of protein concentrates have, to some extent focussed on issues that appear to constrain the cost-effective production of a product. The desired range in protein content for an aquaculture feed product was identified as being between 50 to 70% to allow for a 'one to one' replacement with fishmeal. It appears from this preliminary work that only *Lupinus luteus* (YL) can be considered as having the most suitable attributes warranting further development in the area of lupin concentrates for the feed sector.

13.5 References

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Tables and Figures

Table 13.1 Crude chemical composition (%) estimations of the four lupin species *L. albus*, *L. luteus*, *L. angustifolius*, *L. mutabilis*.

Species	*L. angustifolius*		*L. albus*		*L. luteus*		*L. mutabilis*	
	Seed	Kernel	Seed	Kernel	Seed	Kernel	Seed	Kernel
Seed Coat	24	0	18	0	27	0	16	0
Moisture	9	12	9	11	9	12	8	10
Protein	32	41	36	44	38	52	44	52
Fat	6	7	9	11	5	7	14	17
Ash	3	3	3	4	3	4	3	4
Lignin	1	1	1	1	1	1	1	1
Polysaccharides	22	28	17	21	8	10	9	10
Oligosaccharides	4	6	7	8	9	12	5	6
Minor Components	0.5	1	0.6	1	0.9	1	1	1
Total sum	100	100	100	100	100	100	100	100

Table 13.2 Distribution of NLL cv. Mandelup, kernel flour components via the concentration process.

NLL cv. Mandelup Component	100 g raw wt (g)	Concentrate		Discarded material wt (g)
		wt (g)	% of product	
Protein	46.0	40.9	53.1	5.1
Fat	8.0	6.5	8.4	1.5
Ash	3.5	2.0	2.6	1.5
Lignin	0.7	0.7	0.9	0.0
Kernel polysaccharides	32.8	27.0	35.0	5.8
Oligosaccharides	8.0			8.0
Minor components	1.0			1.0
Total sum	100.0	77.1	100.0	22.9

Table 13.3 Distribution of Yellow lupin cv. Wodjil, kernel flour components via the concentration process.

Yellow lupin cv. Wodjil Component	100 g raw wt (g)	Concentrate		Discarded material wt (g)
		wt (g)	% of product	
Protein	57.5	51.2	71.7	6.3
Fat	8.0	6.5	9.1	1.5
Ash	4.5	3.0	4.2	1.5
Lignin	0.7	0.7	1.0	0.0
Kernel polysaccharides	14.0	10.0	14.0	4.0
Oligosaccharides	14.0			14.0
Minor components	1.3			1.3
Total sum	100.0	71.4	100.0	28.6

Table 13.4 Yield and Final Protein content of both Mandelup and Wodjil kernel flours after various concentration conditions.

NLL cv. Mandelup (starting 46.1% CP)			Concentrate		
Without Preheat Treatment					**Protein**
Temp	Autoclave	%EtOH	Yield %	Protein %	Recovery %
25	no	0	67.7	45.1	73.5
25	no	20	73.6	50.8	89.9
25	no	40	74.7	50.5	90.6
25	no	60	74.8	50.9	91.5
65	no	0	70.2	46.6	78.6
65	no	20	69.3	51.2	85.2
65	no	40	68.8	53.1	87.9
65	no	60	72.0	53.6	92.7
With Preheat Treatment					
25	yes	0	69.5	53.7	89.7
25	yes	20	72.1	52.0	90.0
25	yes	40	73.5	52.6	92.9
25	yes	60	77.0	52.0	96.2
65	yes	0	73.9	51.6	91.6
65	yes	20	66.8	55.7	89.5
65	yes	40	69.2	55.6	92.4
65	yes	60	71.4	54.1	92.8
YL cv. Wodjil (starting 52.0% CP)			**Concentrate**		
Without Preheat Treatment					**Protein**
Temp	Autoclave	%EtOH	Yield %	Protein %	Recovery %
25	no	0	64.9	64.6	80.5
25	no	20	69.7	64.9	86.9
25	no	40	69.6	66.6	89.0
25	no	60	69.9	68.8	92.4
65	no	0	65.4	65.8	82.8
65	no	20	65.3	66.1	83.0
65	no	40	67.2	68.5	88.5
65	no	60	69.1	68.8	91.3
With Preheat Treatment					
25	yes	0	66.6	72.1	92.2
25	yes	20	69.2	70.1	93.3
25	yes	40	70.1	68.3	92.0
25	yes	60	72.9	65.0	91.1
65	yes	0	72.1	67.6	93.6
65	yes	20	64.0	68.7	84.5
65	yes	40	67.2	69.6	89.8
65	yes	60	68.4	69.9	92.0

Table 13.5 The distribution of protein, fat and carbohydrates in the Fibre, Protein Isolates 1 and 2 extracted via 'standard extraction' Figure 2.

NLL cv. Myallie		Fibre		Protein Isolate 1		Protein Isolate 2		Material lost
Kernel flour (dry basis)	WT(g)	WT(g)	% of product	WT(g)	% of product	WT(g)	% of product	WT(g)
Protein	46.0	8.0	20.4	27.0	91.5	4.5	75.0	6.5
Fat	8.0	1.5	3.8	1.5	5.1	0.5	8.3	4.5
Ash	3.5	2.0	5.1	1.0	3.4	0.2	3.3	0.3
Lignin	0.7	0.7	1.8				0.0	0.0
Kernel polysaccharides	32.8	27.0	68.9			0.8	13.3	5.0
Oligosaccharides	8.0							8.0
Minor components	1.0							1.0
Total sum	100.0	39.2	100.0	29.5	100.0	6.0	100.0	25.3

Table 13.6 Protein extraction efficiency of NLL cv. Myallie, (Wongan Hills, 2001) using different treatments.

	NLL cv. Myallie	Kernel Meal	Fibre		Isolate 1		Isolate 2		Fibre + Isolates 1+ 2	
ID	Treatments	% CP	WT Yield %	% CP	WT Yield %	% CP	WT Yield %	% CP	WT Yield %	% CP recovery
Treatments group A: Coarse kernel meal particle size; 23 per cent < 500 μm										
C	Control	42.1	52.7	30.8	19.3	88.1	5.0	73.8	77.0	87.7
A1	Acid wash	42.1	52.6	31.0	18.9	90.1	2.4	77.3	73.9	83.6
A2	Overnight Soak	42.1	41.5	22.7	15.7	89.9	6.1	75.3	63.4	66.9
A3	Overnight Freeze	42.1	45.9	28.5	17.2	88.4	6.0	72.6	69.1	77.5
A4	70 Degree Celsius	42.1	51.1	32.5	18.8	83.4	6.3	78.5	76.2	88.4
A5	pH 12	42.1	51.0	28.6	22.6	86.2	4.3	76.5	77.9	88.7
Treatments group B: Fine kernel meal particle size; 83 per cent < 500 μm										
C	Control	42.1	46.7	25.7	23.0	91.3	6.0	74.8	75.7	91.7
B1	Pectinase (2 h)	42.1	47.4	23.7	14.3	89.0	11.0	80.4	72.7	77.9
B2	Pectinase (16 h)	42.1	42.1	21.8	14.6	85.8	10.6	74.7	67.3	70.4
B3	Acetate buffer	42.1	41.0	18.8	18.6	82.2	14.6	71.8	74.2	79.5
B4	pH 9 (16 h)	42.1	35.8	15.7	29.4	81.7	12.3	75.3	77.5	92.5
Treatments group C: Kernel meal particle size variation										
C1	Coarse	42.1	53.0	30.8	19.3	88.1	5.0	73.8	77.3	87.9
C2	Fine	42.1	49.0	25.0	23.3	86.2	7.3	78.4	79.6	90.4
C3	Flour	42.1	32.0	14.4	30.3	91.2	8.0	75.6	70.3	90.9

Table 13.7 Mass balance data of the protein extraction efficiency of five lupin species.

Species	Coarse Kernel meal	Fibre		Protein Isolate 1		Protein Isolate 2		Fibre + Isolates 1 + Isolate 2	
	% CP	WT Yield %	% CP	WT Yield %	% CP	WT Yield %	% CP	Weight recovery %	CP (%) recovery
NLL (Myallie)	42.1	52.7	30.8	19.3	88.1	5.0	73.8	77.0	87.7
Albus (K. Mutant)	50.0	46.3	30.2	24.5	88.6	4.4	74.9	75.2	78.0
YL (Wodjil)	51.6	43.0	39.1	25.3	88.6	5.8	74.5	74.1	84.4
Mutabilis	50.3	43.7	41.8	28.6	83.1	3.1	72.3	75.4	88.0

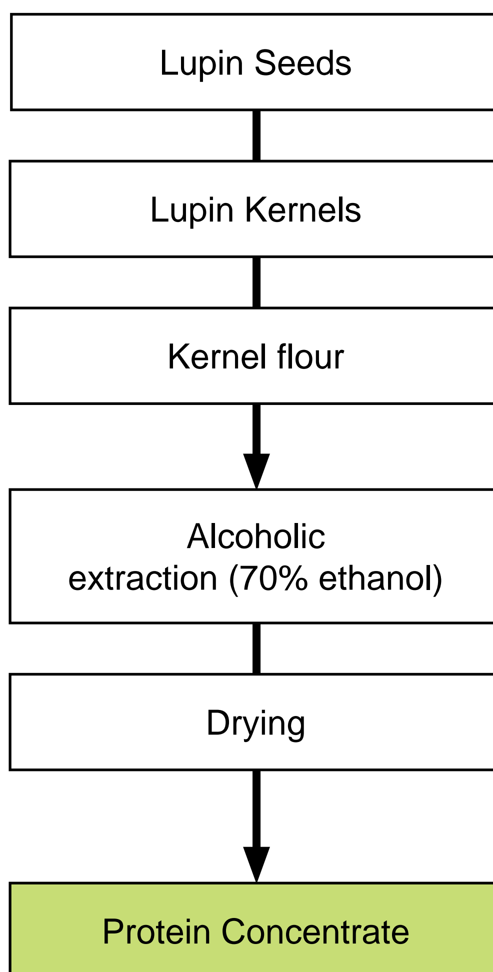

Figure 13.1 Schematic flow chart for the production of lupin concentrate.

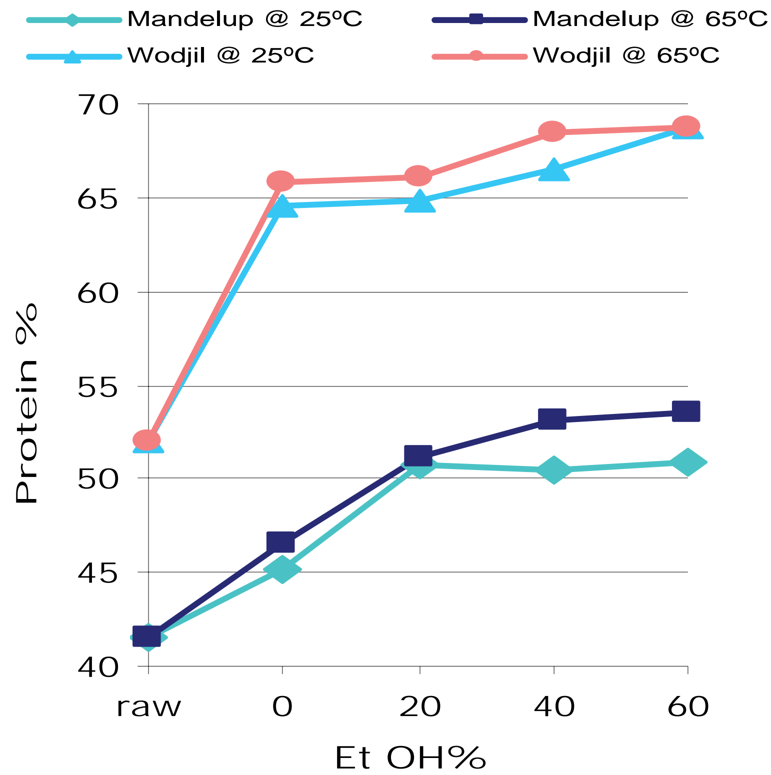

Figure 13.2 Protein concentrations achieved from Mandelup and Wodjil lupin kernel flours with and without preheat treatment washed with a range of ethanol concentrations (0-60%) at 65°C and 25°C.

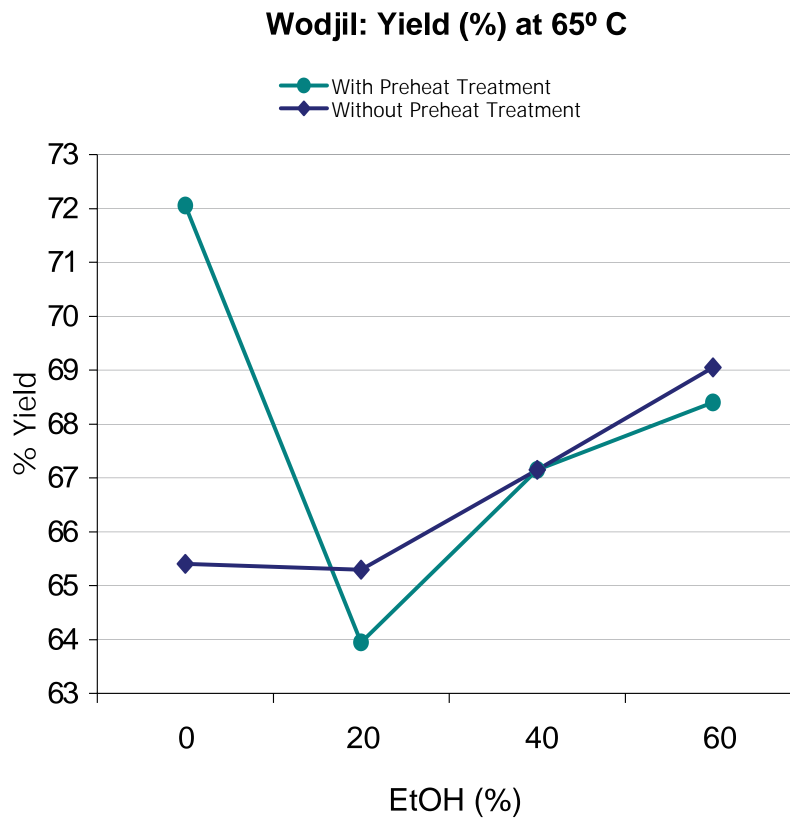

Figure 13.3. Yield achieved from yellow lupin cv. Wodjil lupin kernel flours with and without preheat treatment washed with a range of ethanol concentrations (0-60%) at 65°C.

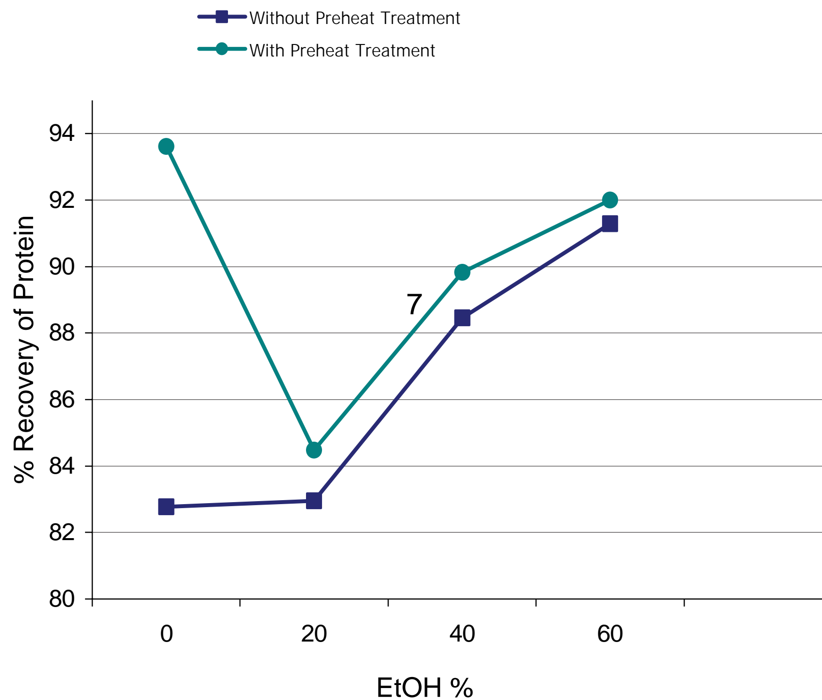

Figure 13.4 Protein recovery achieved from two Wodjil lupin kernel flours with and without preheat treatment washed with a range of ethanol concentrations (0-60%) at 65°C.

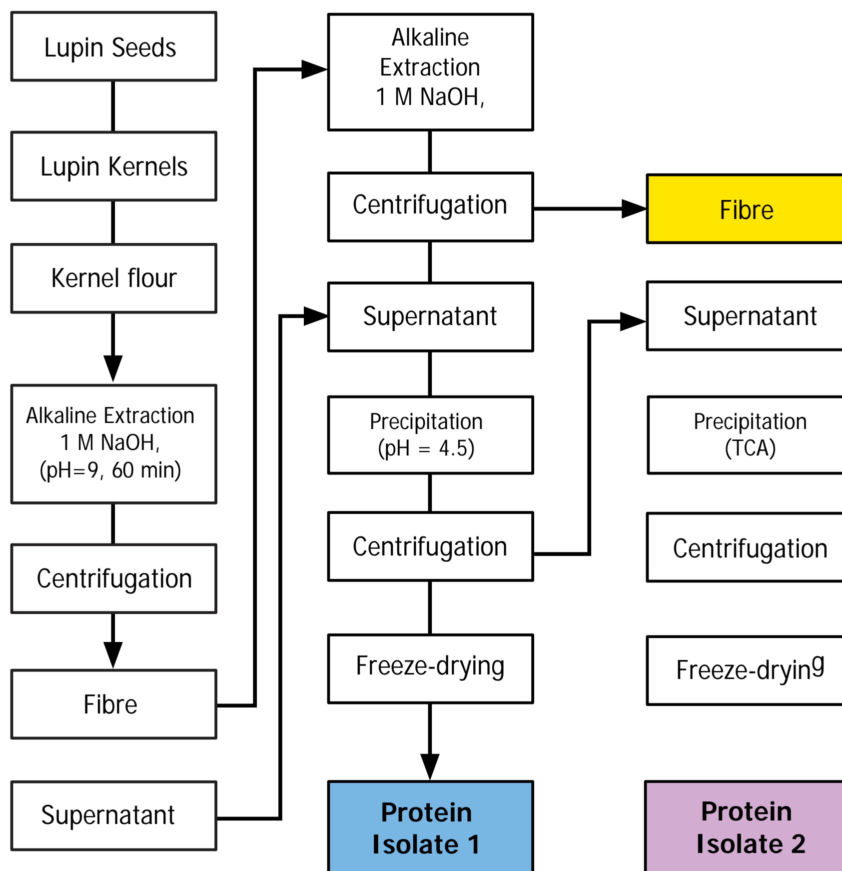

Figure 13.5 Flow diagram for the production of Lupin Fibre, Protein Isolate 1 and Protein Isolate 2.

14.0 Evaluation of the digestible value of lupin and soybean protein concentrates and isolates when fed to rainbow trout, *Oncorhynchus mykiss*, using either stripping or settlement faecal collection methods[a]

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Abstract

A series of prototype protein concentrate and isolate products were prepared from the kernel meals of *Lupinus angustifolius* (cv. Gungarru) and *L. luteus* (cv. Wodjil). The digestible value of these value-added meals, the original kernel meals and a range of similar soybean based products were compared when fed to rainbow trout (*Oncorhynchus mykiss*). Both faecal stripping and settlement collection methods were used to allow a comparison of the effects of these collection methods on the determination of digestible energy and nutrient values of the component ingredients being tested. Significant differences were observed on the digestibility of component ingredients between the two faecal collection methods with the faecal stripping collection method was the more conservative of the two assessments. This was also principally related to the significant differences observed on nutrient and energy digestibilities of the same ingredients between the two faecal collection methods, particularly those ingredients higher in carbohydrates. Both faecal collection methods evaluated demonstrated substantial nutritional value in all of the products evaluated. Significant improvements in most digestible parameters were observed with increasing levels of processing of both lupin species and soybean meal. The largest relative increase in digestibilities of organic matter, energy and protein were seen between the kernel meals and protein concentrates. Improvements with further protein isolation, from concentrate products to isolate products were limited. Phosphorus digestibilities of all lupin products were very high and in contrast to the other nutrient digestibilities diminished with increasing levels of lupin processing. Significant effects on faecal integrity were also noted among the grain products.

14.1 Introduction

Modern nutrient-dense diets for aquatic species have little formulation flexibility to accommodate large amounts of non-useful nutritional content (e.g. fibre or ash). Because of this, many plant protein resources are not viable alternatives, despite having reasonable protein or energy digestibilities. To address this limitation one option is to process some grain varieties to produce protein concentrate or protein isolate products. Such protein concentrated products also allow some flexibility to remove potential antinutritional factors found in plant meals (Glencross et al., 2003a).

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Techniques for production of protein concentrates and isolates from legumes are relatively well known. Among these are processes such as dehulling, air-classifying, solvent extraction and solubilised extraction (Lasztity et al., 2001), all of which have some commercial application.

Notably, a range of such products produced from soybean exist in the market already and have previously been assessed in rainbow trout and Atlantic salmon (Kaushik et al., 1995; Refstie et al., 1998). While there is a growing volume of work examining the use of lupin kernel meals in diets for rainbow trout and Atlantic salmon, there are few studies that have examined the value of both lupin protein concentrates and isolates (Carter and Hauler, 1999; Burel et al., 2000; Glencross and Hawkins, 2004; Glencross et al., 2004a; 2004b).

There are several key facets to determining the nutritional or biological value of a feed ingredient, principal of which is defining the proportion of nutrients that an animal can obtain from a particular ingredient through its digestive and absorptive processes. One of the key methods that can be used to determine the discrete nutrient and energy digestibility of a component ingredient is the diet-substitution method (Aksnes et al., 1996). This method relies on the comparison of a test diet with that of a reference diet. Substantial refinements have also been made to this assessment method through calculation of digestibilities relative to nutrient contribution rather than gross ingredient contribution (Sugiura et al., 1998).

It is well known that the faecal collection method can influence the digestibility assessment of a diet (Vandenberg and de la Noue, 2001), but it is not clear if this difference also means that the component ingredient digestibilities are also different or remain the same through similar relative differences in the overall digestibilities of the test and reference diets. This study compares the digestible value of a variety of protein concentrates and isolates prepared from *L. angustifolius* and *L. luteus* meals with a range of similar soybean products, fishmeal and enzymatically-hydrolysed casein, when fed to rainbow trout, *Oncorhynchus mykiss*. In this study both stripping and settlement methods were used for collecting faeces and a comparison is made of the effects of these two collection methods on the determination of component ingredient digestibilities. This study shared faecal settlement data from earlier work in which some of the diets used in this study were also evaluated in Atlantic salmon to examine differences between these fish species (Glencross et al., 2004b).

14.2 Materials and Methods

14.2.1 Ingredient and diet development

The kernel meals of the lupin species; *Lupinus angustifolius* (cv. Gungarru) and *L. luteus* (cv. Wodjil) were used in this study. Protein concentrates and isolates were made from each kernel meal variety using soluble extraction and filtration techniques. Alkali solution solubilised the protein content of the kernel meals, which was then filtered to remove the insoluble carbohydrate content, before the solution was acidified to precipitate the protein. The protein was allowed to settle before decanting then freeze-dried. The key difference in processing method between the concentrates and isolates was the fineness of the filtration mechanism used to remove non-solubilised components. A comprehensive outline of the protein extraction methods used is reported in Lasztity et al. (2001). The composition and source of all of the ingredients used are presented in Table 14.1. Each of the test ingredients was thoroughly ground such that it passed through an 800 µm square-holed sieve.

The experiment design was based on a diet formulation strategy that allowed for the diet-

substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 160 g/kg DM fat and an inert marker (chromic oxide at 15 g/kg) (Table 14.2). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 14.2). Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 3 mm diameter die. The resultant moist pellets were then oven dried at 90°C for approximately 6 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. The source of all of the ingredients used is presented in Table 14.2. Composition of all experimental diets is presented in Table 14.3.

14.2.2 Fish handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Ward et al., 2003) were transferred from grow-out ponds to experimental tanks (250 L). Freshwater (salinity < 1 PSU) of 22.1 ± 1.8°C at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 6 trout of 266 ± 18 g (mean ± S.D.; n = 15). Treatments were randomly assigned amongst 48 tanks, with each treatment having four replicates.

Fish were hand fed the diets daily to apparent satiety as determined over three separate feeding events between 1800 and 1900. The trout were allowed to acclimatise to the allocated dietary treatment for six days before faecal collection commenced (Wybourne and Carter, 1999). Faeces were collected using both stripping and settlement techniques. Stripping techniques were based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mLl/L) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not contaminated by urine and mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial on ice and later stored in a freezer at -20°C. Stripped faeces were collected during 0800 to 1200 over a six-day period.

Settled faeces were also collected overnight from the same tanks and fish using settlement methods based on those reported by Cho and Kaushik (1990). Faeces were removed from an ice-chilled collection tube at 0700 on each day, prior to the fish being stripped. Faeces were stored at -20°C in between collection periods.

Faecal samples from different days were pooled within collection method and tank, and kept frozen at -20°C before being freeze dried in preparation for analysis.

14.2.3 Chemical and digestibility analysis

All chemical analyses were contracted to professional analytical service providers (Chemistry Centre, East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, chromium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 100°C for 24 h. Total chromium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by Hillebrand et al. (1953). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the Soxhlet method. Gross ash content was

determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein or gross energy to chromium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Cr_{diet} \times Parameter_{faeces}}{Cr_{faeces} \times Parameter_{diet}} \right)$$

where Cr_{diet} and Cr_{faeces} represent the chromium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 13.4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%.

14.2.4 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Effects of ingredient on digestibility of organic matter, nitrogen, phosphorus and gross energy in each of the diet were examined by two-way ANOVA with faecal collection method and ingredient set as key factors (Table 14.5). Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

14.3 Results

Some of the settlement data used for comparisons in this study has been previously reported in another study (Glencross et al., 2004b). The settlement data in this paper is further expanded with some additional ingredients not previously reported. The stripping data has not been previously reported.

14.3.1 Ingredient composition

The lupin based ingredients produced in this study had a range of compositions (Table 14.1). The lupin protein isolates (LPI and API) had protein levels greater than 800 g/kg DM, which was

less than that of the soy protein isolate (893 g/kg DM), but notably they had significantly higher level of lipids (123 to 125 g/kg DM). The lupin protein concentrate products (LPC and APC) had significantly different levels of protein (781 and 690 g/kg DM respectively) and both were significantly higher than that of the soy protein concentrate (590 g/kg DM). In addition, the lupin protein concentrates also had higher level of lipids (78 to 93 cf. 54 g/kg DM). Consistent with the differences in the protein level of the two lupin protein concentrate, the two lupin kernel meals (LKM and AKM) also had significantly different protein levels (547 and 415 g/kg DM).

14.3.2 Comparison of collection methods

Significant differences between the two collection methods were noted on the digestibilities of both the diets and the ingredients studied (Tables 14.4 and 14.6). Greatest influence of collection method was noted on the assessment of phosphorus digestibilities. Typically, phosphorus digestibilities were higher with stripping as the faecal collection method, though there were notable exceptions to this observation (Table 14.4). The least amount of significant differences between collection methods was noted among the energy digestibilities.

The greatest difference between collection methods on ingredient assessment was noted for the sweet lupin kernel meal (AKM), which had each parameter significantly different between the two collection methods. The digestibility of the yellow lupin kernel meal (LKM) was also significantly affected by collection method, with only energy digestibility not discerned as different between the two collection methods. The digestibility assessment of several ingredients was unaffected by faecal collection methods. Notable among these ingredients were fishmeal, soy protein concentrate and enzymatically-hydrolysed casein. An interaction effect of collection method and ingredient was also noted for each of the response variables.

14.3.3 Ingredient assessment based on stripping collection

Based on faecal samples collected using stripping techniques, organic matter digestibilities of the ingredients varied substantially (Table 14.6). Notably the organic matter digestibility of the AKM was the poorest (44.6%) and SPI the most digestible (96.4%) of all the ingredients evaluated. The total levels of digestible organic matter were lowest for the AKM (431 g/kg DM) and SPI the highest (919 g/kg DM) (Table 14.7). Phosphorus digestibility of the soybean meal was the poorest (27.7%) and phosphorus from the AKM the most digestible (346.0%) of all the ingredients evaluated. The total levels of digestible phosphorus were lowest for the soybean meal (2 g/kg DM) and equally highest for the fish meal and EHC (8 g/kg DM) (Table 14.7). Energy digestibility of the AKM was the poorest (53.1%) and the fish meal the most digestible (99.0%) of all the ingredients evaluated. The total levels of digestible energy were lowest for the AKM (10.8 MJ/kg DM) and SPI the highest (22.0 MJ/kg DM) (Table 14.7). The nitrogen digestibility of the AKM was the poorest (85.3%) and the LPC the most digestible (102.1%) of all the ingredients evaluated. The total levels of digestible organic matter were lowest for the AKM (354 g/kg DM) and SPI the highest (877 g/kg DM) (Table 14.7).

Some significant differences of faecal integrity were also noted among the diets used in the faecal collection study (Table 14.8). The most distinct and well formed faeces were observed from fish fed the LPC, Basal and Fishmeal diets. The most diffuse and least well formed faeces were from fish fed the diets containing the SPI, SPC, API, APC and LKM diets.

14.3.4 Ingredient assessment based on settlement collection

Based on faecal samples collected using settlement techniques, organic matter digestibilities

of the ingredients also varied substantially (Table 14.5, 14.6 and 14.7). Notably the organic matter digestibility of the AKM was the poorest (64.8%), though not significantly poorer than that of soybean meal, and EHC the most digestible (98.5%) of all the ingredients evaluated. The total levels of digestible organic matter were lowest for the AKM (627 g/kg DM) and API the highest (920 g/kg DM) (Table 14.7). Phosphorus digestibility of the fishmeal was the poorest (36.2%) and phosphorus from the AKM the most digestible (272.2%) of all the ingredients evaluated. The total levels of digestible phosphorus were equally lowest for a range of ingredients, including AKM, LPC, APC, API and SPI (4 g/kg DM) and equally highest for the fish meal and EHC (8 g/kg DM) (Table 14.7). Energy digestibility of the AKM was the poorest (70.5%) and the EHC the most digestible (98.8%) of all the ingredients evaluated. The total levels of digestible energy were lowest for the AKM (14.4 MJ/kg DM) and SPI the highest (21.4 MJ/kg DM) (Table 14.7). The nitrogen digestibility of the fish meal was the poorest (89.3%) and the SPC the most digestible (106.9%) of all the ingredients evaluated. The total levels of digestible nitrogen were also lowest for the AKM (403 g/kg DM) and highest for the SPI (873 g/kg DM) (Table 14.7).

14.4 Discussion

The comparison of the influence of faecal collection method on the determination of the digestible value of a range of lupin and soybean based products highlights not only the considerable potential of these protein resources for use in aquaculture diets, but also the importance of faecal collection method on the assessment of their digestible value. Although studies have been performed comparing the determination of whole diet digestibilities based on faeces collected using either settlement of stripping techniques (Vandenberg and de la Noue, 2001), this is the first study to compare the influence of these faecal collection techniques on the component digestibility assessment of test ingredients. This study builds on data presented in Glencross et al. (2004b).

14.4.1 Faecal collection methods

Irrespective of the debate on the positives and negatives associated with either collection method, it is acknowledged that the two faecal collection methods do result in different digestibility value determinations (Vandenberg and de la Noue, 2001). The present study also demonstrates that this difference also extends to the determination of the component ingredient digestibilities. An example of this shown in Figure 14.1. This difference implies that the differences in whole diet digestibilities are not necessarily relative within faecal collection methods. This difference is important in that it suggests that the assessment of ingredient digestibilities needs also to be considered in context of what faecal collection method was used.

Of the two faecal collection methods it could be argued that ingredient digestibility determinations from faeces collected using settlement are potentially overestimations of true digestibility, while those determinations from faeces collected using stripping techniques are underestimations of true digestibility. Clearly the faecal stripping collection method presents as the more conservative of the two assessments.

The greatest differences between the nutrient digestibility assessments from the two faecal collection methods were noted on those ingredients with higher levels of indigestible carbohydrates, such as the *L. angustifolius* and *L. luteus* kernel meals and soybean meal. The most pronounced nutrient effects were those on organic matter digestibilities, though effects on energy, phosphorus and nitrogen digestibilities were also noted. It was noted however that

there were limited differences in digestibility assessment between faecal collection methods for ingredients with low carbohydrate contents, such as fish meal, soybean protein isolate and enzymatically hydrolysed casein. We suggest that the presence of high levels of carbohydrates in the faeces increases the dissolution of the faecal matter when expelled into the water column, thereby effectively reducing the total nutrient content of the faeces collected and consequently inflating the digestibility value determined from those samples. An assessment of organic matter digestibility from the two different faecal collection methods as a function of ingredient nitrogen-free extractive content shows clear relationships (Figure 14.1). This was also largely reflected in energy digestibilities. The relationship between nitrogen and phosphorus digestibilities was not consistent with NFE content of the ingredients. Certainly, in furthering the development of plant protein resources for use in aquaculture feeds, there are merits in pursuing faecal stripping as the preferred faecal collection method.

14.4.2 Grain protein product evaluation

In the present study a range of grain products of varying processing levels were evaluated. Included in this assessment were products from two lupin species, *L. angustifolius* cv. Gungarru and *L. luteus* cv. Wodjil, and soybeans. A high-quality fish meal and enzymatically hydrolysed casein were also included as reference ingredients. Notably, each of the prototype protein concentrate and isolate products produced from lupins was in fact made from the same batches of the original kernel meals. The soybean products were not from a definable background and were obtained from three separate sources.

Within faecal collection method used, a variety of differences in digestible values were noted among the different products. With the progressive removal of carbohydrate material from both series of the lupin products improvements in digestibility of organic matter, energy and nitrogen/protein were observed. It is well recognised that lupin carbohydrates are predominantly non-starch polysaccharides (NSP) and constitute limited nutritional value for most monogastric animals (van Barneveld, 1999). These observations are also consistent with those reported by Glencross et al. (2003b), who noted that even within *L. angustifolius* kernel meals that there was a strong relationship between kernel meal protein content and protein digestibility. Similar such improvements were also noted in the present study amongst the soybean products. These observations are consistent with that reported by others evaluating soy protein products (Kaushik et al., 1995; Refstie et al., 1998).

In contrast to the general improvements seen in most digestibility parameters with increasing level of product processing, there was a relative deterioration of the digestibilities of phosphorus from the lupin products with increasing removal of the carbohydrate content of each meal. However, it should be noted that in each case the phosphorus digestibilities were still assessed as greater than 100%. We believe this effect to be due to the low phosphorus content of these products enforcing the fish to further derive phosphorus from the reference diet to a greater degree than that achieved in the reference diet treatment. The result of this increased digestion of phosphorus from the reference component of each test diet being that the calculated effect on phosphorus digestibility of the products to be greater than 100%. Rationally, this cannot be the case, but the observation is still important to note because it recognises the influence of ingredient combinations on nutrient digestibilities. Accordingly it is more logical to report digestible nutrient contents of each ingredient in such a case as 100% x ingredient P content. This is presented in Table 14.7.

14.4.3 Conclusions

The two faecal collection methods used in this study are the two main methods used by fish nutritionists worldwide and this study provides a good estimate of how well each method assesses component ingredient digestibilities. The faecal stripping collection method is the more conservative of the two assessments. In furthering the development of plant protein resources in particular, there are merits in using faecal stripping as the preferred faecal collection method, because of its inherent conservatism. This was also principally related to the significant differences observed between the two faecal collection methods, particularly those higher in carbohydrates.

Independent of the faecal collection methods, substantial nutritional value was found in all of the products evaluated in this study. Significant improvements in most digestible parameters were observed with increasing processing of both lupin species and soybean meal. The largest relative increase in digestible value was seen between the kernel meals and protein concentrates. Improvements with further protein isolation, from concentrate products to isolate products were limited. To further develop such products for use in the aquaculture sector a focus needs to be made on determining levels of product palatability and inherent nutritional value through nutrient utilisation studies.

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Table 14.1 Nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[a] Pregel starch	[c] LKM	[d] AKM	[e] LPC	[f] APC	[g] LPI	[h] API	[i] Soybean meal	[j] SPC	[k] SPI	[l] EHC
Dry matter content (g/kg)	917	906	903	885	944	942	924	926	909	939	938	916
Crude protein	770	7	547	415	781	690	805	810	518	590	893	839
Crude fat	68	11	87	53	78	93	123	125	47	54	13	11
Ash	142	3	44	33	37	31	41	30	69	79	47	70
Phosphorus	22	0	6	4	6	5	9	5	8	9	9	9
Organic matter	858	997	956	967	963	969	959	970	931	921	953	930
Gross energy (MJ/kg DM)	21.3	17.2	20.9	20.4	22.2	22.2	22.6	22.6	19.6	20.3	23.0	21.2
Lysine	45.7	0.6	22.5	13.9	31.6	24.8	28.6	24.5	27.6	28.3	45.5	59.5
Threonine	31.9	1.7	19.9	16.0	24.2	23.0	24.2	22.8	23.8	25.3	35.7	39.5
Methionine	21.1	0.0	4.2	2.6	5.1	4.8	5.0	3.8	8.8	8.5	12.7	24.7
Isoleucine	28.4	2.1	19.5	15.4	27.6	27.0	27.7	28.1	22.6	25.8	38.3	46.2
Leucine	54.8	0.0	44.5	29.1	62.9	50.8	65.6	50.6	43.9	47.7	71.7	78.8
Valine	34.0	0.0	18.5	14.4	24.8	23.3	24.3	24.3	23.6	27.0	40.2	61.0
Phenylalanine	29.4	0.0	21.1	16.0	30.4	27.8	30.5	28.0	27.2	30.2	45.1	40.5
Histidine	24.6	0.0	14.7	10.3	18.2	15.4	19.4	16.2	14.1	15.0	22.2	23.7
Arginine	43.2	0.0	61.1	47.2	79.3	77.9	85.9	81.2	41.7	44.9	68.3	33.0

[a] Fish meal: Chilean anchovy meal, Skretting Australia, Cambridge, TAS, Australia. [b] Pregelatinised wheat starch, Weston Biotechnologies, Henderson, WA, Australia. [c] LKM: Yellow lupin: *L. luteus* Kernel Meal, Coorow Seed Cleaners, Coorow, WA, Australia. [d] AKM: Sweet lupin: *L. angustifolius* Kernel Meal, WESFEEDS, Bentley, WA, Australia. [e] LPC: *L. luteus* Protein Concentrate, Department of Agriculture, South Perth, WA, Australia. [f] APC: *L. angustifolius* Protein Concentrate, Department of Agriculture, South Perth, WA, Australia. [g] LPI: *L. luteus* Protein Isolate, Department of Agriculture, South Perth, WA, Australia. [h] API: *L. angustifolius* Protein Isolate, Department of Agriculture, South Perth, WA, Australia. [i] SPC: Soy Protein Concentrate, Hamlet Protein HP300, Copenhagen, Denmark. [j] SPI: Soy Protein Isolate, ICN Biomedical, Costa Mesa, CA, USA . [l] EHC: Enzymatically Hydrolyzed Casein, ICN Biomedical, Costa Mesa, CA, USA

Table 14.2 Formulations of the experimental diets (all values are g/kg).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
Fishmeal	650	755	455	455	455	455	455	455	455	455	455	455
Fish oil	110	77	77	77	77	77	77	77	77	77	77	77
[a]Luteus Kernel Meal (LKM)	0	0	300	0	0	0	0	0	0	0	0	0
[b]Angustifolius Kernel Meal (AKM)	0	0	0	300	0	0	0	0	0	0	0	0
[a]Luteus Protein Concentrate	0	0	0	0	300	0	0	0	0	0	0	0
[b]Angustifolius Protein Concentrate	0	0	0	0	0	300	0	0	0	0	0	0
[a]Luteus Protein Isolate	0	0	0	0	0	0	300	0	0	0	0	0
[b]Angustifolius Protein Isolate	0	0	0	0	0	0	0	300	0	0	0	0
Soybean	0	0	0	0	0	0	0	0	300	0	0	0
Soy Protein Concentrate	0	0	0	0	0	0	0	0	0	300	0	0
Soy Protein Isolate	0	0	0	0	0	0	0	0	0	0	300	0
Enzymatically Hydrolyzed Casein	0	0	0	0	0	0	0	0	0	0	0	300
Pregelled starch	150	105	105	105	105	105	105	105	105	105	105	105
Cellulose	65	46	46	46	46	46	46	46	46	46	46	46
Vitamin and mineral premix*	10	7	7	7	7	7	7	7	7	7	7	7
Chromic oxide	15	11	11	11	11	11	11	11	11	11	11	11

[a] From L. luteus (yellow lupins). [b] From L. angustifolius (Sweet lupins).

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Table 14.3 Nutrient composition of the experimental diets (all values are g/kg DM, unless otherwise indicated).

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12
Dry matter content (g/kg)	963	957	952	962	951	961	953	961	959	947	962	951
Crude protein	476	551	496	457	568	546	582	577	492	521	600	580
Crude fat	174	153	147	146	134	110	135	132	129	128	121	126
Ash	108	118	90	87	87	114	93	85	100	101	91	97
Nitrogen-free extractives	241	178	267	310	211	230	189	206	279	250	188	197
Organic matter	858	956	967	963	969	959	970	931	921	953	930	858
Phosphorus	16	18	14	14	12	12	13	12	13	13	13	13
Gross energy (MJ/kg DM)	22.64	22.37	22.33	22.13	22.50	22.41	22.51	22.60	21.86	21.88	22.52	22.19

Table 14.4 Digestibility (%) of all experimental diets by two faecal collection methods.

	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8	Diet 9	Diet 10	Diet 11	Diet 12	Pooled SEM
Stripping													
Organic Matter	83.4	86.3	75.4	71.3	86.4	82.0	85.5	84.8	76.8	78.7	87.7	85.2	0.84
Phosphorus	41.5	38.4	58.2	61.7	58.1	55.7	47.7	52.8	41.0	50.3	46.2	54.2	1.32
Energy	85.5	88.9	78.8	76.0	88.6	85.6	87.5	87.4	81.4	86.2	89.5	87.3	0.79
Nitrogen	91.3	91.9	90.7	89.9	95.6	93.1	93.4	92.8	90.9	91.7	94.6	92.6	0.36
Settlement													
Organic Matter	85.8	88.4	84.4	79.3	88.5	85.7	87.7	88.8	83.6	84.9	88.9	89.8	0.45
Phosphorus	47.2	42.3	61.7	60.2	53.5	54.5	49.5	51.7	51.2	51.4	48.6	57.6	0.88
Energy	87.6	89.5	85.7	82.2	89.4	87.7	88.7	89.6	85.9	87.2	90.1	90.8	0.37
Nitrogen	93.9	94.2	95.3	95.0	96.0	95.7	95.7	95.8	94.9	96.4	95.9	95.7	0.13

Table 14.5 Two-way ANOVA table for statistical parameters of faecal collection method and ingredient, with additional one-way ANOVA tables for ingredient differences within fish species.

	Parameter	SS	DoF	MS	F	p
Method	Organic matter	0.184	1	0.184	26.918	0.000
Ingredient	Organic matter	1.544	10	0.154	22.548	0.000
Method x Ingredient	Organic matter	0.141	10	0.014	2.066	0.040
Method	Phosphorus	1.256	1	1.256	11.297	0.001
Ingredient	Phosphorus	50.426	10	5.042	45.336	0.000
Method x Ingredient	Phosphorus	1.898	10	0.189	1.707	0.098
Method	Energy	0.046	1	0.04653	3.679	0.059
Ingredient	Energy	0.976	10	0.09764	7.719	0.000
Method x Ingredient	Energy	0.133	10	0.01332	1.053	0.410
Method	Nitrogen	0.039	1	0.039	15.45	0.000
Ingredient	Nitrogen	0.155	10	0.015	6.100	0.000
Method x Ingredient	Nitrogen	0.039	10	0.004	1.530	0.148
		SS	**DoF**	**MS**	**F**	**p**
Stripping						
Ingredient	Organic matter	1.236	10	0.124	10.075	0.000
	Phosphorus	32.352	10	3.235	19.280	0.000
	Energy	0.859	10	0.086	3.647	0.002
	Nitrogen	0.121	10	0.012	2.582	0.019
Settlement						
Ingredient	Organic matter	0.449	10	0.045	31.530	0.000
	Phosphorus	19.972	10	1.997	36.543	0.000
	Energy	0.250	10	0.025	14.410	0.000
	Nitrogen	0.072	10	0.007	19.800	0.000

SS: Sum of squares. DoF: Degrees of Freedom. MS: Mean squares.

Table 14.6 Digestibility (%) specifications of the test ingredients.

Nutrient	Fishmeal	LKM	AKM	LPC	APC	LPI	API	Soybean meal	SPC	SPI	EHC	Pooled SEM
Stripping												
Organic matter	${}^{a}93.1^{a}$	${}^{a}57.5^{bc}$	${}^{a}44.6^{c}$	${}^{a}92.8^{a}$	${}^{a}70.7^{b}$	${}^{a}88.3^{a}$	${}^{a}87.6^{a}$	${}^{a}61.0^{b}$	${}^{a}67.2^{b}$	${}^{a}96.4^{a}$	${}^{a}89.1^{a}$	2.95
Phosphorus	${}^{a}35.1^{de}$	${}^{a}183.3^{b}$	${}^{a}346.0^{a}$	${}^{a}131.5^{bc}$	${}^{a}138.5^{bc}$	${}^{a}67.5^{de}$	${}^{a}120.9^{cd}$	${}^{a}27.7^{e}$	${}^{a}76.3^{d}$	${}^{a}54.0^{de}$	${}^{a}92.3^{cd}$	14.15
Energy	${}^{a}99.0^{a}$	${}^{a}64.2^{c}$	${}^{a}53.1^{c}$	${}^{a}94.4^{a}$	${}^{a}84.2^{ab}$	${}^{a}92.4^{ab}$	${}^{a}91.3^{ab}$	${}^{a}72.1^{bc}$	${}^{a}87.3^{ab}$	${}^{a}95.6^{a}$	${}^{a}91.5^{ab}$	2.94
Nitrogen/Protein	${}^{a}87.5^{b}$	${}^{a}88.6^{b}$	${}^{a}85.3^{b}$	${}^{a}102.1^{a}$	${}^{a}98.4^{a}$	${}^{a}99.4^{a}$	${}^{a}95.1^{ab}$	${}^{a}92.1^{b}$	${}^{a}97.9^{a}$	${}^{a}98.2^{a}$	${}^{a}92.2^{b}$	1.21
Settlement												
Organic matter	${}^{a}94.5^{ab}$	${}^{b}80.9^{c}$	${}^{b}64.8^{c}$	${}^{a}94.1^{ab}$	${}^{a}76.7^{c}$	${}^{a}90.0^{b}$	${}^{a}94.8^{a}$	${}^{b}77.3^{c}$	${}^{b}82.0^{c}$	${}^{a}95.2^{a}$	${}^{a}98.5^{a}$	1.62
Phosphorus	${}^{a}36.2^{d}$	${}^{a}175.9^{b}$	${}^{b}272.2^{a}$	${}^{b}70.7^{c}$	${}^{b}87.2^{c}$	${}^{b}52.9^{cd}$	${}^{b}71.7^{c}$	${}^{a}56.7^{cd}$	${}^{a}58.9^{cd}$	${}^{a}42.2^{cd}$	${}^{a}85.4^{c}$	10.73
Energy	${}^{a}96.4^{ab}$	${}^{b}83.8^{d}$	${}^{b}70.5^{e}$	${}^{a}92.3^{bc}$	${}^{a}86.6^{cd}$	${}^{a}91.7^{bc}$	${}^{a}93.8^{ab}$	${}^{a}83.3^{d}$	${}^{a}85.6^{d}$	${}^{a}93.1^{ab}$	${}^{a}98.8^{a}$	1.27
Nitrogen/Protein	${}^{a}89.3^{d}$	${}^{b}97.2^{c}$	${}^{b}97.2^{c}$	${}^{a}99.3^{bc}$	${}^{a}101.0^{b}$	${}^{a}101.1^{b}$	${}^{a}98.6^{bc}$	${}^{a}99.0^{bc}$	${}^{b}106.9^{a}$	${}^{a}97.8^{c}$	${}^{a}96.0^{c}$	0.67

Different pre superscripts within columns indicate significant differences between means of collection method but within nutrients and ingredients ($P < 0.05$).

Different post superscripts within rows indicate significant differences between means among ingredients, but not between nutrients or collection method ($P < 0.05$).

Table 14.7 Digestible nutrient content (g/kg DM, unless otherwise detailed) of the test ingredients.

Nutrient	Fishmeal	LKM	AKM	LPC	APC	LPI	API	Soybean meal	SPC	SPI	EHC
Stripping											
Organic matter	799	550	431	893	685	847	850	568	619	919	828
Phosphorus	8	6	4	6	5	6	5	2	7	5	8
Energy (MJ/kg DM)	21.1	13.4	10.8	21.0	18.7	20.9	20.6	14.1	17.7	22.0	19.4
Nitrogen/Protein	673	485	354	781	679	800	770	477	578	877	774
Settlement											
Organic matter	811	774	627	907	743	863	920	720	756	907	916
Phosphorus	8	6	4	4	4	5	4	5	5	4	8
Energy (MJ/kg DM)	20.5	17.5	14.4	20.5	19.2	20.7	21.2	16.3	17.4	21.4	20.9
Nitrogen/Protein	688	531	403	776	690	805	799	513	590	873	805

Digestible nutrient values are calculated based on ingredient composition (Table 1) and ingredient apparent digestibility coefficients (Table 4). Where apparent digestibility coefficients were greater than 100%, an absolute digestibility of 100% was assumed for practicality reasons.

Table 14.8 Integrity of faeces as stripped from rainbow trout.

Diet	Treatment	Mean	SEM
1	Basal	3.1 [a]	0.4
2	Fishmeal	3.1 [a]	0.2
3	LKM	2.4 [b]	0.2
4	AKM	2.7 [ab]	0.1
5	LPC	3.3 [a]	0.1
6	APC	2.4 [b]	0.1
7	LPI	3.0 [ab]	0.1
8	API	2.4 [b]	0.1
9	Soybean	2.7 [ab]	0.2
10	SPC	2.4 [b]	0.2
11	SPI	2.4 [b]	0.2
12	EHC	2.7 [ab]	0.1

Faecal integrity based on the following subjective scaling: 1 – liquid faeces only; 2 – Watery faeces, no form, but not totally liquid; 3 – Faecal form developing, but no distinct faecal pellets; 4 – Distinct faecal pellets; 5 – Firm, dryish, punctuated distinct faecal pellets. Different superscripts indicate significant differences.

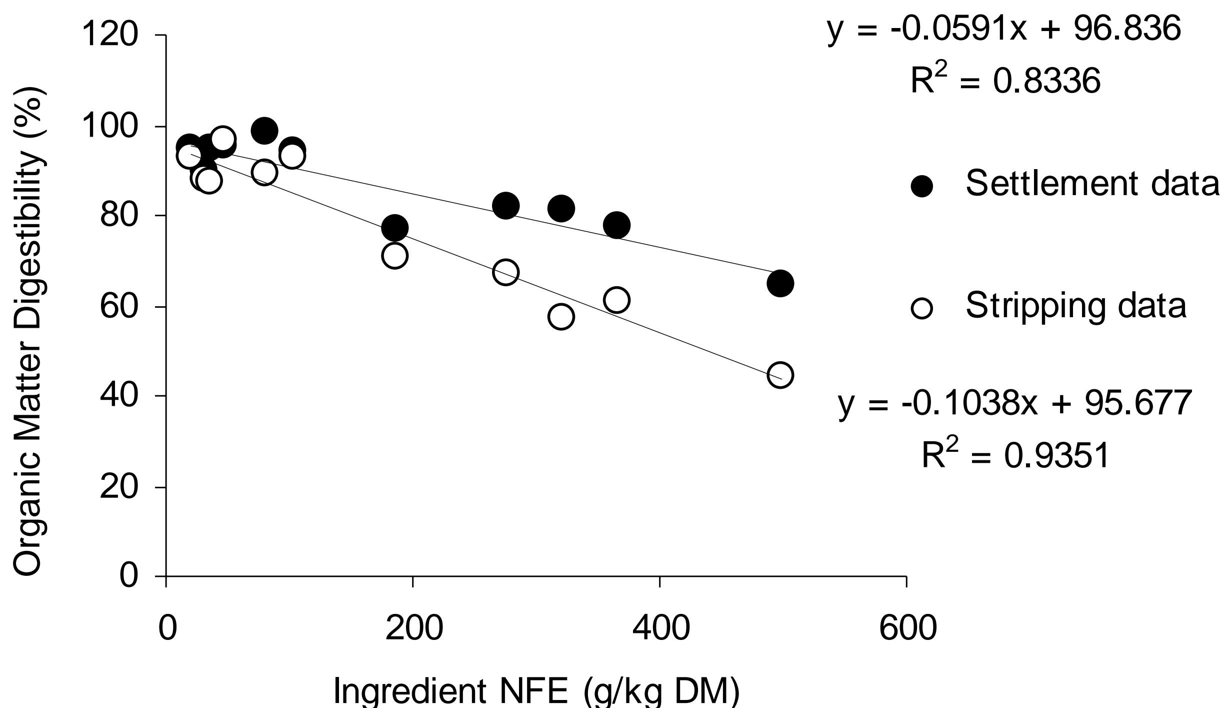

Figure 14.1 Organic matter (OM) digestibility of the ingredients, using either faecal collection method, as a function of Nitrogen-Free Extractive (NFE) content of the ingredients. Both collection methods provide similar estimates at low NFE levels, but at higher levels the OM digestibility are more conservative from faeces collected using stripping techniques.

15.0 An evaluation of the digestible value of value-added lupin (*Lupinus angustifolius, L. luteus* and *L. mutabilis*) products produced using concentrate or isolate technologies when fed to rainbow trout (*Oncorhynchus mykiss*)

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Abstract

A series of value-added grain products were produced from the kernel meals of three lupin species of *Lupinus angustifolius, L. luteus* and *L. mutabilis*. Products were made using either extractive or isolation techniques to produce a concentrate or isolate from each grain respectively. The value-added products were then included in diets at a 300 g/kg inclusion level to assess their apparent dry matter, protein and energy digestibilities. It was observed that use of the extractive value-adding techniques only marginally increased the protein content of *L. angustifolius* kernel meal, but that a more significant increase in protein content was observed using extractive techniques on the *L. luteus* and *L. mutabilis* kernel meals. The use of protein isolation techniques substantially increased the resultant protein content of all products produced from each of the *L. angustifolius* and *L. luteus* lupins species. Assessment of the digestible dry matter, protein and energy from each of the value-added grain products demonstrated that the *L. angustifolius* protein concentrate (APC) produced using extractive methods actually had a reduced level of digestible dry matter, protein and energy compared to its starting kernel meal. Although the protein digestibility of both the *L. luteus* and *L. mutabilis* protein concentrate products produced using extractive methods were substantially better than that of the APC, their protein digestibility was still not as high as that of the grain-product produced using isolation techniques from the same grain. This work demonstrates that the technique used to produce a value-added product not only affects its chemical composition, but that it can also affect its digestible value. Protein isolation was shown to be a more robust method for both increasing protein levels and also maintaining the nutritional value of the grain products.

15.1 Introduction

There is an ongoing need to reduce the reliance on the use of fishmeal as a protein source in aquaculture feeds. In order to reduce the risks associated with being reliant on any single raw material type, be that economic, supply or quality issues there is an imperative to increase the range of raw materials available for use in aquaculture feeds. To address this risk, substantial work has been undertaken to assess alternative ingredients for use in aquaculture feeds (Glencross et al., 2007b).

There has been a particular focus on the nutritional value to fish of grain products produced from soybean, peas and lupins as alternative feed ingredients, where the grain has been processed to

produce a dehulled product (Kaushik et al., 1995; Refstie et al., 1998; Carter and Hauler, 2000; Burel et al., 2000; Glencross and Hawkins, 2004; 2004b), concentrated product or isolated product (Glencross et al., 2004a; 2005; Refstie et al., 2006).

Of most of the grain products studied, it has been noted that the protein content in these products tends to be at the lower ranges of useful levels in being able replace significant levels of fishmeal (Glencross et al., 2007b). Therefore it would be of value if these grains could be processed to enhance their protein content and thereby increase the flexibility with which they might be used in aquaculture feeds. There has been some assessment of a range of products produced from either soybeans or lupins, and of various protein concentrations (Kaushik et al., 1995; Carter and Hauler, 2000; Booth et al., 2001; Glencross et al., 2004a; 2005).

Most grains also possess some level of intrinsic anti-nutritional factors (ANF) (Francis et al., 2001; Glencross et al., 2003; 2006b). The opportunity to slightly enhance the nutritional characteristics of grains through grain processing also lends itself the potential to remove any ANFs. An example of this was the work on the development of a series of prototype protein concentrates from lupin kernel meals of varying compositional characteristics has been produced using isolation techniques was examined (Lasztity et al., 2001; Glencross et al., 2006a).

The processing technique used to manufacture a value-added grain product can affect not only its chemical composition, but also its nutritional value (Glencross et al., 2004c; 2004d; Glencross et al., 2007a). Damage to protein quality, as well as the removal or retention of nutritionally non-useful or useful material, is always a possibility with any processing method. Therefore, following any pilot processing method it is prudent to not only assess the chemical composition, but also the new products nutritional value.

In evaluating the potential of feed ingredients there are several ways to determine the nutritional or biological values, principal of which is defining the proportion of nutrients and energy that an animal can obtain from a particular ingredient through its digestive and absorptive processes (Glencross et al., 2007b). Only once a raw material's digestible value has been defined can robust, balanced diets be formulated to provide meaningful growth response data from animals to which the ingredients are then fed.

As a preliminary way of evaluating a new series of ingredients, this study examines the digestibility of a series value-added grain products. The products were produced from a range of lupin species (*Lupinus angustifolius, L. luteus* and *L. mutabilis*) using either extractive or isolation protein concentration methods and the effects of the different processing methods on their nutritional value to rainbow trout, *Oncorhynchus mykiss* are examined.

15.2 Materials and Methods

15.2.1 Ingredient development

Samples of *L. angustifolius* cv Myallie and *L. luteus* cv Wodjil kernel meals were obtained from a commercial grain processor. Samples of the *L. mutabilis* seed were obtained from the Department of Agriculture and Food, Western Australia's lupin-breeding program, dehulled and milled to create stock samples *L. mutabilis* kernel meal. Dehulling was conducted as described in Glencross et al. (2007c). From each of the lupin kernel meals either or both protein isolates and concentrates were prepared as the amount of material permitted.

Protein concentrates from *L. angustifolius, L. luteus* and *L. mutabilis* were prepared by

cooking the flours (autoclaved) at 122°C for 60 min (inclusive of ramp up, ramp down and depressurisation). Following cooking the flours were sieved through a 300 µm sieve with the addition of water to produce a 15:1 mix of water to flour. This mix was stirred for 60 min before being filtered through a 50 µm filter bag. The residue was then washed again in water (15:1, water : residue) before being filtered for a second time through a 50 µm filter bag. The remaining residue was then frozen at -20°C prior to being freeze-dried. Following the freeze-drying process, each of the PC's was re-milled to ensure all particles were < 800 µm particle size. The *L. mutabilis* had two additional washing steps to ensure removal of alkaloids.

Protein isolates from *L. angustifolius* and *L. luteus* were prepared from samples of each meal that were solubilised in water at room temperature and the pH adjusted to 9.0 with NaOH (1.0 M) with vigorous stirring for 60 min. After mixing, the solution was filtered through an 800 µm filter bag to separate the non-solubilised material from the solubilised protein. The protein solution was then brought to a pH of 4.5 with the addition of HCl (1.0 M) to precipitate out the solubilised protein whilst held at 4°C. The protein precipitate was decanted and dried in a freeze drier. The extraction processes are based on those reported in Lasztity et al. (2001). Following the freeze-drying process, both of the PI's was re-milled to ensure all particles were < 800 µm particle size.

The composition and source of all of the ingredients used are presented in Table 15.1. Each of the test ingredients was thoroughly ground such that they passed through a 750 µm hammer mill screen. Not all ingredients were assessed in digestibility studies due to material and equipment constraints.

15.2.2 Diet development

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 15.2). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 15.2). Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. The diet formulations and source of all of the ingredients used is presented in Table 18.2. Composition of all experimental diets is also presented in Table 15.2.

15.2.3 Fish handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 L). Freshwater (salinity < 1 PSU) of 16.1 ± 0.3°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 15 trout of 361 ± 43.7 g (mean ± S.D.; n = 40). Treatments were randomly assigned amongst 24 tanks, with each treatment having three replicates.

Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600 each day. The trout were allowed to acclimatise to the

allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was taken to ensure that the faeces were not contaminated by urine or mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and stored in a freezer at -20°C. Stripped faeces were collected during 0800 to 1000hrs over a four-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples from different days were pooled within tank, and kept frozen at -20°C before being freeze-dried in preparation for analysis.

15.2.4 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia and Animal Health Laboratories, South Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by McQuaker et al., (1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Amino acid composition of samples was determined by acid hydrolysis prior to separation via HPLC. The acid hydrolysis destroyed tryptophan making it unable to be determined. Crude fat content of the diets was determined gravimetrically following extraction of the lipids according to the method of Folch et al. (1957). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

where Y_{diet} and Y_{faeces} represent the yttrium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 15.4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$

and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%.

15.2.5 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Effects of ingredient on digestibility of dry matter, protein and gross energy in each of the ingredient were examined by one-way ANOVA (Table 15.3). Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

15.3 Results

15.3.1 Ingredient composition

The ingredients produced in this study, were from one of three different species of lupin seed and had a range of compositions (Table 15.1). The protein isolation process in contrast to the protein concentration process had a clear significant effect of increasing protein content and reducing carbohydrate content of the products. Only a marginal increase in protein content was observed between the *L. angustifolius* kernel meal (AKM) and protein concentrate (APC) (Table 15.1). More significant gains were achieved in protein content through the isolation process (e.g. API). A substantially greater increase in protein content was observed between the *L. luteus* kernel meal and protein concentrate (LPC) (Table 15.1). Accordingly, differences in the protein content between the LPC and the *L. luteus* protein isolate (LPI) were less.

Protein concentrates were typically lower in crude fat than both the kernel meals and the protein isolates (Table 15.1).

15.3.2 Diet digestibility

Apparent dry matter digestibilities of the diets increased (> 0.835) with inclusion of the protein isolates although a decline in diet digestibility was observed with the inclusion of the soybean meal (SBM), AKM, APC, LPC and MPC (Table 15.3). Apparent protein digestibilities of the diets increased (> 0.909) with the addition of all grain products except the APC and LPC (Table 15.3). Apparent energy digestibilities of the diets generally declined (< 0.910) with the addition of all grain products (Table 15.3).

15.3.3 Ingredient digestibility

Apparent dry matter digestibilities of the value-added grain products generally improved with increasing protein content across most grain varieties (Table 15.3). An exception to this was the dry matter digestibility of the APC, which was lower than that of the AKM. The API also had higher dry matter digestibility than the LPI, despite having lower combined protein and fat

levels. The API had the highest (0.901) dry matter digestibility of all the products evaluated and the APC the lowest (0.405) (Table 15.3).

Apparent protein digestibilities of the value-added grain products were largely unaffected by the increased protein content of the value-adding processes (Table 15.3). Indeed a significant decline in protein digestibility was observed between the AKM and APC. The APC also had a significantly lower protein digestibility than the API, but the same observation was not consistent between the LPC and the LPI. Protein digestibility of the MPC was similar to that of the LPC and LPI, but both were lower than that of the API. The AKM had the highest protein digestibility (0.992) of all products evaluated and the LPC the lowest (0.903) (Table 15.3).

Apparent energy digestibilities of the value-added grain products were significantly improved by the increased protein content of the value-adding processes (Table 15.3). Although a significant decline in energy digestibility was observed between the AKM and APC. The APC also had a significantly lower energy digestibility than the API, but the same observation was not as consistent to the same degree between the LPC and the LPI. The energy digestibility of the MPC was similar to that of the LPI, and was higher than that of both he APC and LPC. The API had the highest energy digestibility (0.884) of all products evaluated and the APC the lowest (0.585) (Table 15.3).

15.4 Discussion

There are an increasing number of studies examining the digestible value of lupins and lupin products when fed to a variety of fish species (Burel et al., 1998; Booth et al., 2001; Glencross and Hawkins, 2004; Glencross et al., 2004a; 2004b; 2005; 2006a; 2007a). Although most of these studies have focussed on the nutritional assessment of lupin kernel meals, there is also an increasing capacity for the potential use of other valued added products like protein concentrates and isolates (Glencross et al., 2005; 2007a). While the advantages of dehulled versus whole seed lupins have been made clear across a range of species (Booth et al., 2001; Glencross et al., 2007c), further benefits may be obtained by using products with higher protein levels still, so as to provide greater relief from fish meal and also increase diet formulation flexibility.

15.4.1 Ingredient composition

The ingredients produced in this study, produced from one of three different species of lupin, had a range of compositions consistent with the potential range in protein contents observable between lupin kernel meals and protein isolates as reported in other studies (Glencross et al., 2005; 2006a; 2007a). The protein isolation process as has been observed in other studies, was far more successful in concentrating the protein, but it is notably a less efficient process with much lower yields. That only a marginal increase in protein content was observed between the *L. angustifolius* kernel meal (AKM) and protein concentrate (APC) would also raise the question concerning if the cost associated with such an extractive value-adding process would be recouped in the value of the final product. Clearly more significant gains were achieved in protein content through the isolation processes for all grain varieties studied and this process also appears to produce a more nutrient dense product. However, the composition of the “ideal” specifications for such a value-added grain product for the aquaculture sector are difficult to define precisely, as they will depend on a variety of factors such as cost and availability of other alternatives and also the cost and efficiency of any value-adding processes used. (Glencross, 2003).

It is interesting that the protein concentrates were typically lower in crude fat than both the

kernel meal and the protein isolates from their respective lupin varieties. This supports that the extractive processes used to prepare the concentrates also removed a significant component of the kernel meal lipid. While removal of the lipid can be regarded as a value-adding process through the redirection of the lipid to other uses, as in some sectors such as the soybean industry, in this case it has substantially reduced the nutritional value of the protein concentrate from a compositional perspective.

15.4.2 Ingredient digestibilities and nutritional value

Significant improvements in most digestible parameters were observed with increasing levels of protein concentration of the different lupin varieties. The key exception to this was the digestible value of the protein concentrates APC, LPC and MPC, which despite increases in their protein content had reduced relative values of that protein and also their energy content. It is suspected that this may have occurred through damage to the nutritional value of the protein in these value-added products during the autoclaving process during their manufacture, similar to what was reported in Glencross et al. (2004c; 2004d; 2007b) from the application of heat in the drying process.

The combined effect of the protein and energy digestibilities (Table 15.3) and the composition of the different products (Table 15.1) are combined to derive the digestible values of each the products presented in Table 15.3. By comparison of the digestible dry matter, protein and energy values it is possible to deduce the nutritional value derived from the various components in each ingredient. For example, the soybean and *L. angustifolius* kernel meal both have similar levels of digestible protein (464 g/kg DM vs 409 g/kg DM), but the soybean has a markedly higher dry matter digestibility (616 g/kg DM vs 438 g/kg DM). This suggests, that based on the fact that there is limited lipid in the soybean meal and the similarity of the energy digestibility of the two grains, that substantial amounts of the soybean carbohydrates are absorbed, while those of the lupin kernel meal are not. This observation is consistent with other reports on the digestibility of soybean and lupin kernel meals when fed to trout (Kaushik et al. 1995; Glencross et al., 2005).

Another interesting comparison is that between the AKM and the APC (Table 15.3). Given that the APC is derived from the extractive processing of the AKM, it can be noted that there is a net decline in the digestible dry matter, protein and energy of the APC. The AKM had a digestible protein level of 409 g/kg DM, while the APC had a digestible protein level of 372 g/kg DM. The digestible energy declined from 12.3 to 12.1 MJ/kg DM also. This supports that the processes used to produce the APC have in fact deteriorated its nutritional value as a feed product for fish. Possible reasons for this may be that much of the protein has been damaged, reducing its digestible value (Glencross et al., 2004), or that the processing has changed the nutritional profile of what is there to increase the level of fibre in the ingredient as has been observed in other studies (Glencross et al., 2004c; 2007a).

A comparison among the protein isolates produced from each lupin variety (API and LPI) show that irrespective of starting material, this value-adding process consistently produces products of the highest protein content and most consistent digestible value (Table 15.1 and 15.3). The process also retains significant amounts of lipid. However, the high digestible protein content probably exceeds that needed for a bulk-commodity required to replace substantial amounts of fish meal in aquaculture diets.

15.4.3 Conclusions

This study confirms that there are some compositional and nutritional benefits to the manufacture of lupin protein concentrates and isolates. The use of extractive concentrating technology was not as reliable or robust as that of isolating technology in both protein yield and also the quality of the protein produced. Notably, despite increases in protein content through concentrating processes, in some cases a reduction in the net digestible protein was achieved. This in effect would reduce the value of the protein concentrated product too less than that of the original starting material.

The use of *L. mutabilis* as a new grain variety also shows some promise, though further assessment of its nutritional value as a kernel meal would perhaps be more appropriate.

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Table 15.1 Nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[a] Wheat	Soybean meal	AKM	APC	API	LKM	LPC	LPI	MKM	MPC
Dry matter content (g/kg)	931	905	899	916	918	937	909	939	931	900	939
Crude protein	749	142	497	412	500	754	537	719	819	554	759
Crude fat	87	24	21	97	69	153	77	62	112	189	87
Ash	161	11	75	35	13	23	44	14	29	44	13
Phosphorus	28	2	8	5	3	7	7	3	7	-	3
Gross energy (MJ/kg DM)	20.5	18.4	19.9	20.6	20.7	25.1	21.1	22.0	24.2	24.2	23.1
Arginine	39	7	35	43	48	84	53	66	83	49	65
Cysteine	9	4	9	7	8	12	16	20	24	9	16
Histidine	18	1	12	9	12	17	14	17	18	13	20
Isoleucine	33	5	23	16	24	35	21	29	31	20	34
Leucine	60	10	42	29	43	64	44	64	72	32	61
Lysine	51	5	32	13	22	33	27	27	36	19	31
Methionine	26	2	9	4	5	6	5	8	7	3	8
Phenylalanine	30	6	27	16	23	34	22	29	33	17	29
Threonine	37	5	24	17	23	32	19	30	29	18	35
Valine	39	7	23	14	20	29	17	25	26	17	28

[a] Wheat and Fish meal: Chilean anchovy meal, Skretting Australia, Cambridge, TAS, Australia. SBM: Solvent-extracted soybean meal: WESFEEDS, Bentley, WA, Australia. AKM: *L. angustifolius* kernel meal: Coorow Seed Cleaners, Coorow, WA, Australia. APC: *L. angustifolius* protein concentrate, API: *L. angustifolius* protein isolate. LKM: *L. luteus* kernel meal, LPC: *L. luteus* protein concentrate, LPI: *L. luteus* protein isolate, MKM: *L. mutabilis* kernel meal, MPC: *L. mutabilis* protein concentrate: Department of Agriculture and Food – Government of Western Australia, South Perth, WA, Australia..

Table 15.2 Formulations of the experiment diets (all values are g/kg).

	Reference Diet	SBM	AKM	APC	API	LPC	LPI	MPC
Ingredient								
Fishmeal	700.0	490.0	490.0	490.0	490.0	490.0	490.0	490.0
Fish oil	150.0	105.0	105.0	105.0	105.0	105.0	105.0	105.0
Solvent-Extracted Soybean meal		300.0						
L. angustifolius kernel meal			300.0					
L. angustifolius concentrate				300.0				
L. angustifolius isolate					300.0			
L. luteus concentrate						300.0		
L. luteus isolate							300.0	
L. mutabilis concentrate								300.0
Wheat flour	144.0	100.8	100.8	100.8	100.8	100.8	100.8	100.8
Vitamin and mineral premix*	5.0	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1.0	0.7	0.7	0.7	0.7	0.7	0.7	0.7
Diet composition as analysed								
Dry matter	961	964	952	949	962	946	960	958
Protein	494	498	478	504	575	565	586	574
Fat	233	172	186	185	195	182	179	190
Carbohydrate**	149	222	239	221	139	163	142	146
Phosphorus	19	15	14	14	15	14	15	14
Ash	124	108	97	90	90	90	93	90
Gross Energy	22.9	21.7	22.4	22.4	23.4	22.8	23.0	23.0

SBM: Solvent-extracted soybean meal, AKM: *L. angustifolius* kernel meal, APC: *L. angustifolius* protein concentrate, API: *L. angustifolius* protein isolate. LPC: *L. luteus* protein concentrate. LPI: *L. luteus* protein isolate. MPI: *L. mutabilis* protein isolate.

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

**Carbohydrate determined as dry matter minus protein, fat and ash.

Table 15.3 Digestibility (%) specifications of diets and test ingredients and digestible nutrient content (g/kg DM, unless otherwise detailed) of the test ingredients as determined using stripping faecal/digesta collection methods.

Nutrient	Reference	SBM	AKM	APC	API	LPC	LPI	MPC	Pooled SEM
Diet Digestibility									
Dry matter	0.835 ^{a}	0.775 ^{b}	0.728 ^{c}	0.709 ^{c}	0.847 ^{a}	0.777 ^{b}	0.833 ^{a}	0.818 ^{ab}	0.011
Protein	0.909 ^{c}	0.910 ^{c}	0.914 ^{c}	0.896 ^{d}	0.933 ^{a}	0.902 ^{d}	0.924 ^{b}	0.918 ^{b}	0.002
Energy	0.910 ^{a}	0.854 ^{b}	0.816 ^{c}	0.814 ^{c}	0.909 ^{a}	0.858 ^{b}	0.905 ^{a}	0.891 ^{ab}	0.008
Ingredient Digestibility									
Dry matter	–	0.685 ^{c}	0.478 ^{b}	0.405 ^{a}	0.901 ^{e}	0.620 ^{c}	0.853 ^{d}	0.789 ^{d}	0.021
Protein	–	0.933 ^{b}	0.992 ^{a}	0.917 ^{c}	0.977 ^{ab}	0.903 ^{c}	0.921 ^{c}	0.931 ^{bc}	0.007
Energy	–	0.648 ^{c}	0.595 ^{c}	0.585 ^{c}	0.884 ^{a}	0.762 ^{b}	0.855 ^{a}	0.848 ^{a}	0.019
Digestible Nutrients									
Dry matter	–	616	438	372	844	582	794	741	
Protein	–	464	409	458	737	649	754	707	
Energy (MJ/kg DM)	–	12.9	12.3	12.1	22.2	16.8	20.7	19.6	

SBM: Solvent-extracted soybean meal, AKM: *L. angustifolius* kernel meal, APC: *L. angustifolius* protein concentrate, API: *L. angustifolius* protein isolate. LPC: *L. luteus* protein concentrate. LPI: *L. luteus* protein isolate. MPC: *L. mutabilis* protein concentrate. Different superscripts within rows indicate significant differences between means among ingredients, but not between nutrients or Diet/Ingredient assessment ($P < 0.05$). Digestible nutrient values are calculated based on ingredient composition (Table 15.1) and ingredient apparent digestibility coefficients (Table 4). Where apparent digestibility coefficients were greater than 100%, an absolute digestibility of 100% was assumed for practicality reasons.

16.0 Evaluation of the nutritional value of prototype lupin protein concentrates when fed to rainbow trout (*Oncorhynchus mykiss*)[a]

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Abstract

This study examines the palatability and discrete nutritional evaluation of some prototype lupin protein concentrates (PC) when fed to rainbow trout. Products were developed from both *Lupinus angustifolius* and *L. luteus* kernel meals with an increase in protein of 415 g/kg DM to 690 g/kg DM for *L. angustifolius* and 545 g/kg DM to 750 g/kg DM for *L. luteus* respectively. This study completes a three-phase approach to evaluating the nutritional value of these products. The digestibility of energy, nitrogen, phosphorus and organic matter were determined in earlier studies using the diet substitution approach. The apparent digestibility of the energy from the *L. angustifolius* PC and the *L. luteus* PC, along with the apparent protein digestibility were used to formulate two series of experimental diets to examine both the palatability and discrete nutritional value of the products. Serial inclusion of either PC at 0%, 10%, 20%, 30% and 40% into a typical salmonid diet specification allowed an examination of the palatability of each product. Additional negative-controls, based on the 0% diets with inclusion of sulfamerazine sodium, were included in the experiment to demonstrate the capacity of the experiment to detect significant palatability issues. No significant effects of inclusion of either PC on any fish performance criteria, such as feed intake or growth, were identified. In contrast, significant reductions in feed intake and consequently growth were observed from fish fed either of the negative controls. This experiment demonstrated that each PC was highly palatable at inclusion levels up to and including 40% of the diet. Using a protein-limited-restrictively-fed experimental approach the discrete nutritional utilisation of each PC was defined. Growth of fish fed the PC treatments was not significantly different to that of the 0% reference diet. Two control diets with substitutions of cellulose to an equivalent inclusion level to that of the PC have provided an indication of the net benefit of the test ingredients. This experiment demonstrated that each PC provided equivalent nutritional value to the fish at either of the two inclusion levels (20% and 40%). These PC's differed in their viscosity and gelling properties which may allow feed manufacturers the opportunity to manipulate the physical attributes of their feeds. These studies show that the prototype PC's have substantial potential as a prospective feed ingredient for the aquaculture sector.

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16.1 Introduction

In an effort to reduce reliance on fish meal as their primary protein source most modern, nutrient-dense, aquaculture diets now use some inclusion of plant protein ingredients. Lupin (*Lupinus* spp.) meals are one ingredient that have been shown to provide some potential as a useful feed ingredient in fish diets and are being used in commercial diets in increasing quantities (Burel et al., 1998; Carter and Hauler, 2000; Glencross and Hawkins, 2004).

There are traditionally three lupin species that are commercially produced and used as feed ingredients. These are the European white lupin (*Lupinus albus*), the Australian narrow-leafed lupin (*Lupinus angustifolius*) and the yellow lupin (*Lupinus luteus*) (Petterson, 2000). Typically it is the kernel meals of lupins that are being used in fish diets. This is supported by numerous reports on the nutritional evaluation of all three lupin kernel meal varieties in aquaculture diets (De la Higuera et al., 1988; Gomes et al., 1995; Burel et al., 2000; Farhangi and Carter, 2001; Glencross and Hawkins, 2004; Glencross et al., 2004a).

However, some problems with high inclusion levels of lupins in fish diets have been reported, with minor aberrations in digestion, growth and metabolic processes being reported (Burel et al., 1998; Farhangi and Carter, 2001; Glencross et al., 2004a). These have been attributed to a range of issues including some anti-nutritional factors (Refstie et al., 1998; Francis et al., 2001; Glencross et al., 2003a).

In addition to some issues with prospective ANF in lupin kernel meals it would be of substantial value if they had slightly enhanced nutritional characteristics, such as higher protein levels. To address this, preliminary work on the development of a series of prototype protein concentrates from lupin kernel meals is progressing and a range of products of varying compositional characteristics has been produced (Glencross et al., 2004b). Presently it is unknown if these products have suitable nutritional characteristics for use in aquaculture diets.

In the process of ingredient evaluation there are several key facets to determining or placing a nutritional or biological value on a feed ingredient, principal of which is defining the proportion of nutrients that an animal can obtain from a particular ingredient through its digestive and absorptive processes. Other key facets of this process include the examination of palatability issues and the capacity for the ingredient to be utilised for growth without influence of factors disturbing metabolic utilisation of the diet. In essence this later issue is about determining the extent of any effect of biologically-active components in the ingredient or other factors that might limit its effectiveness as a useful feed ingredient. This strategy has already been used effectively to examine biological value issues in other plant meals (Glencross et al., 2003b). In addition to these biological attributes the influence that an ingredient has on the physical properties of diets is also emerging as an important aspect of ingredient evaluation. Use of rapid-viscosity analysis techniques have been shown useful in this regard and provide a rapid and cost-effective way of examining the variability in functional characteristics of ingredients (Glencross et al., 2004c).

This study reports on the evaluation of the nutritional value of a variety of prototype protein concentrates prepared from lupin (*Lupinus angustifolius* and *L. luteus*) kernel meals when fed to rainbow trout, *Oncorhynchus mykiss*.

16.2 Methods

16.2.1 General methods

16.2.1.1 Ingredients and ingredient preparation

Composition and source of all of the ingredients used are presented in Table 16.1. Lupin kernel meals (*Lupinus angustifolius,* cv. Gungarru and *L. luteus*, cv. Wodjil) were obtained from commercial grain millers and ground to < 800 µm particle size. Samples of each meal were solubilised in water at room temperature and the pH adjusted to 9.0 with NaOH (1.0 M) with vigorous stirring for 60 min. After mixing, the solution was filtered through an 800 µm filter bag to separate the non-solubilised material from the solubilised protein. The protein solution was then brought to a pH of 4.5 with the addition of HCl (1.0 M) to precipitate out the solubilised protein whilst held at 4°C. The protein precipitate was decanted and dried in a freeze drier. The extraction processes are based on those reported in Lasztity et al. (2001). Following the freeze-drying process, both of the PC was re-milled to ensure all particles were < 800 µm particle size. The remaining feed ingredients were obtained as detailed in Table 16.1. This process was used to produce a *Lupinus angustifolius* PC (APC) and a *L. luteus*, PC (LPC).

16.2.1.2 Chemical analysis

Respective samples of diet, faecal and whole-body samples were analysed for a variety of analytes, depending on experiment, including dry matter, ash, fat, nitrogen, phosphorus and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Phosphorus levels were determined using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Kjeldhal digestion, based on N x 6.25. Crude fat content was determined gravimetrically following extraction of the lipids according to the Soxhlet method (AOAC, 1990). Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Organic matter content was determined based on the difference between dry matter content minus ash content. Gross energy was determined by adiabatic bomb calorimetry. Levels of tri-iodothyronine (T_3) and thyroxine (T_4) were determined by a competitive immunoassay method using chemiluminescence detection (Fisher, 1996). For sample analysis parameters, two fish from each replicate were pooled then analysed (n = 3 replicates per treatment).

16.2.2 Ingredient digestibility

The digestibility of the ingredients studied in this paper is reported in Glencross et al. (2005). The digestible protein and energy values from digesta collected from rainbow trout using stripping techniques were used in the calculation of diet digestible protein and energy levels (Table 16.2).

16.2.3 Palatability

16.2.3.1 Diet development

All palatability experiment diets were formulated to be isonitrogenous (400 g/kg) and isoenergetic (19.5 MJ/kg) on a digestible nutrient basis. Digestibility coefficient values for key ingredients were based on those reported earlier. Diets were processed by the addition of water (about 30% of mash dry weight) to all ingredients while mixing to form a dough which was

subsequently screw-pressed through a 4 mm diameter die using a pasta maker. The resultant moist pellets were then oven dried at 90°C for about 9 h before being air-cooled, bagged and stored at -20°C. The antibiotic sulfamerazine sodium, a known feeding deterrent, was added to two diets based on the reference diet, at different levels to create a series of negative controls (Boujard and Le Gouvello, 1997). A commercial extruded salmonid diet was used as the final treatment group. Formulations and proximate composition for all diets are presented in Table 16.3 and 16.4 respectively.

16.2.3.2 Fish management

Forty-eight shallow-conical bottomed 250 L tanks, with flow-through freshwater (4 L/min, salinity < 1 PSU and 16.9 ± 1.3°C, dissolved oxygen 7.5 ± 0.3 mg/L, mean ± SD, n=42), were each stocked with 20 juvenile (9 month, 35.6 ± 0.19 g; mean ± SD) hatchery reared rainbow trout (*Oncorhynchus mykiss;* Pemberton Heat-tolerant Strain). Treatments were randomly assigned in quadruplicate to the tank array. Photoperiod was maintained at 10L: 14D.

The fish were fed to apparent satiety once daily at about 0800 h for 42 days. Apparent satiety, as determined by a loss in feeding activity, was reached after three feeding sessions over a one-hour period. Uneaten feed was removed from each tank one hour later and the uneaten portion dried and weighed to allow the determination of daily feed intake based on correction factors for leaching losses sustained over an equivalent period.

Fish were individually re-weighed after three and six weeks, with all fish within each tank used to determine the average weight gain per tank and treatment (Table 16.5). Five fish were taken as an initial sample for composition analysis. At the end of the study two fish were taken from each tank (4 replicates x 2 fish, per treatment) for whole body analysis. An additional two fish from each tank were sampled for blood biochemistry, within one minute of capture, by caudal tail vein puncture using a 1 mL syringe fitted with at 20G needle. Growth was assessed as mean weight gain and daily growth coefficient (DGC). DGC was calculated as (Kaushik, 1998):

$$DGC = \frac{\left(W_f^{1/3} - W_i^{1/3}\right)}{t} \times 100$$

16.2.4 Nutrient limitation studies

16.2.4.1 Diet development

Test ingredients were included at either 20% or 40% in protein-limited diets that were pair-fed restrictively (PLRF) to all treatments. This design was chosen as it had been previously shown to be useful in examining nutrient utilisation limitations where a focus on the protein source of the diet was important (Glencross et al., 2003c; 2004b). All experiment diets were formulated to be iso-nitrogenous and protein limited (333 g/kg) on a digestible basis. The diets were also formulated to be iso-energetic (15.8 MJ/kg) on a digestible basis. The exceptions to this were the two control diets, where cellulose was added to the diets at equivalent proportions to that of the test ingredients. Digestibility coefficient values for key ingredients were based on those reported earlier. Diets were processed by the addition of water (about 30% of mash dry weight) to all ingredients while mixing to form a dough which was subsequently screw-pressed through a 4 mm diameter die using a pasta maker. The resultant moist pellets were then oven dried at 90°C for approximately 9 h before being air-cooled, bagged and stored at -20°C. Formulations and proximate composition for all diets are presented in Table 16.6 and 16.7 respectively.

16.2.4.2 Fish management and feeding regimes

Experiment conditions were the same as detailed in section 16.2.3.2. Flow-through freshwater (4 L/min, salinity < 1 PSU and 12.5 ± 1.0°C, dissolved oxygen 9.3 ± 0.5 mg/L, mean ± SD, n=42) was provided to each tank, which was stocked with 15 juvenile (12 month, 113.7 ± 1.2 g; mean ± SD) rainbow trout. The fish were fed to a fixed ration based on twice maintenance energy requirements once daily. An additional treatment, using the 0% diet, was fed to satiety (REF-diet) to demonstrate growth potential during the experiment. Care was maintained to ensure almost 100% of all feed offered was consumed however any uneaten feed that was encountered was removed from each tank and accounted for (Table 16.8).

Fish were individually re-weighed after three and six weeks, with all fish within each tank used to determine the average weight gain per tank and treatment (Table 16.8). At the end of the study two fish from each tank were sampled for blood biochemistry, within one minute of capture, by caudal tail vein puncture using a 1 mL syringe fitted with at 20G needle. Growth was assessed as mean weight gain and daily growth coefficient (DGC). Fish composition analysis was not undertaken due to catastrophic sample damage.

16.2.5 Assessment of ingredient pasting characteristics

Samples of the test ingredients were evaluated for their pasting characteristics using a Rapid-Visco-Analyser (RVA; Newport Scientific, Warriewood, NSW, Australia). Samples were added to a dry sample vessel at 3.5 g of dry matter with 22 g of total water content. A standard 1 program (2 min at 50°C, ramping to 95°C over 3 min, hold at 95°C for 5min, before reducing to 50°C for 3 min) was run to examine the pasting characteristics of the added sample. Key features to be examined where the time of gelatinisation, peak viscosity, breakdown viscosity and end viscosity.

16.2.6 Statistical analysis

All figures are mean ± SE unless otherwise specified. Data were analysed for homogeneity of variances using Cochrans test. Effects of diets were examined by ANOVA using the software package Statistica (Statsoft®, Tulsa, OA, USA). Levels of significance were determined using Tukeys HSD test, with critical limits being set at $P < 0.05$. Effects of inclusion level of meal on key performance parameters were examined by linear regression modelling, also using the software package Statistica.

16.3 Results

16.3.1 Ingredient palatability

16.3.1.1 Feed intake and efficiency effects

Significant differences between treatments in palatability were determined based on daily feed consumption over the first ten days and cumulative feed consumption over the term of the experiment. Daily intakes of both control diets with sulfamerzine sodium were significantly less consumed than all other diets (Figure 16.1). The 40% inclusion of APC also resulted in less daily feed intake than the other treatments (Figure 16.1). Cumulative feed consumption only showed significant differences in feed intake for the sulfamerzine sodium negative controls, although a markedly lower amount of the 20% APC diet was also consumed over the study compared to the other treatments (Table 16.5).

Feed utilisation efficiency (as food conversion ratio; FCR or food conversion efficiency; FCE) was not significantly affected by the inclusion of either LPC or APC (Table 16.6). Feed utilisation efficiency of the two control diets was also not significantly different from the reference or treatment diets.

16.3.2 Growth effects

Weight gain by the fish from experiment 1 was largely consistent with the patterns of feed intake (Table 16.5). Weight gain by fish for both of the sulfamerzine sodium negative controls was significantly lower compared to the other treatments (Table 16.5). No other significant weight gain differences were observed. Nutrient (nitrogen and phosphorus) and energy retention was significantly improved with the dietary inclusion of either LPC or APC (Table 16.5). Nutrient retention by the fish was largely unaffected in the negative controls, although a significant decline in energy retention was observed at the highest inclusion of sulfamerzine sodium, consistent with the low feed intakes observed in this treatment. There were no significant effects of treatments on fish survival, which was greater than 95% for all treatments.

16.3.3 Nutrient utilisation

The results from the PLRF trial showed that the discrete nutritional value of the test ingredients in amino acid and energy balanced diets were not significantly different from that of fish meal (Table 16.9). In addition, the APC and LPC products had equivalent discrete nutritional value as both the soy protein concentrate (S) and the *L. luteus* kernel meal (K). Comparison of the 20% inclusion test diets with the 20%C (cellulose) diets showed the net value of that specific ingredient to the diet when fed to the fish. Similarly, the comparison of the 40% inclusion test diets with the 40%C (cellulose) diets again showed the net value of that specific ingredient at those higher inclusion levels. There was no significant effect of treatments on fish survival, which was greater than 95% for all treatments.

Feed intake was not significantly different amongst any of the test diets or the 20%C diet, but the feed intake of the reference (REF-diet) treatment was significantly higher and the feed intake of the 40%C treatment was significantly lower than all other treatments (Table 14.9). Feed use efficiency (FCR or FCE) was not significantly different among any of the 20% or 40% inclusion test treatments. The feed efficiency of both the cellulose diets (20%C and 40%C) was both significantly higher than the test ingredients. There were no significant differences in feed efficiency between the reference diet fed restrictively or to satiety (Table 16.8).

Data variability for the blood thyroid hormones was high. However, there were several significant differences among the test ingredients with respect to the concentrations of the thyroid hormone, tri-iodothyronine (T_3) in the blood of the fish (Table 16.9). Fish fed the 40%A diet had significantly higher levels of T_3 than the fish fed the 20%A or the 0% diets, but were not significantly different from any of the other treatments. There were no significant differences in the levels of thyroxine (T_4) among any of the treatments.

16.3.4 RVA pasting characteristics

Examination of the pasting characteristics of the two protein concentrates and the respective kernel meals from which they were made showed clear differences in the functional properties of the different products (Figure 16.2). The APC showed a lower initial viscosity than the AKM, with the AKM showing earlier gelling characteristics than the APC. The final viscosities of the products increased though the analysis and at the end were both similar. Both the LPC and

LKM products had substantially lower viscosities than either the APC or AKM products. As with the APC/AKM products, the increased concentration of the protein, and lower levels of carbohydrates reduced the initial viscosity of the products, although the final viscosity was similar (Table 16.1 and Figure 16.2).

16.4 Discussion

The focus of these studies has been the comparison of the nutritional value of two new prototype lupin protein concentrates when fed to rainbow trout. Principal in defining the nutritional value of a particular ingredient is the examination of the influence of the ingredient on the animal's digestive and absorptive processes. Traditionally this has been addressed through digestibility studies (Cho and Slinger, 1979). Each of the ingredients studied in the present paper have already been evaluated for their digestible nutrient and energy value and are reported in Glencross et al. (2005). This study follows on from that earlier work and reports the assessment of the palatability, nutrient utilisation value and functional property assessment of those same products.

16.4.1 Palatability effects

The use of plant protein products in aquaculture diets is sometimes limited by the effects of the ingredients on the palatability of the diets when fed to the fish (Gomes et al., 1995; Burel et al., 1998). Because of this key effect it is important to evaluate the relative effects that specific feed ingredients have on the feed intake by the target species. However, it is also recognised that diet energy density also has an effect on feed intake and therefore it is important that digestible energy density of all diets is maintained constant (Kaushik, 1998). The results from the palatability study demonstrated that the APC product initially caused feed intake problems at its highest inclusion levels (40%). However, by day 42 of the experiment, the cumulative feed intake of the 40% APC diet was not significantly different from any of the other treatments, and neither was the growth achieved by the fish fed that diet. In contrast, the LPC product had no palatability issues at any of the tested inclusion levels. Based on these observations it was supported that both of the lupin protein concentrate products were highly palatable to rainbow trout, with only minor palatability problems noted at the highest inclusion levels of the APC product. These findings are consistent with those reported by others that have also not observed a decline in feed intake of lupin products fed at even higher than 40% inclusion levels (Farhangi and Carter, 2001; Glencross et al., 2004a).

16.4.2 Differences in discrete nutritional value between plant protein ingredients

The findings of these two studies show that provided that the diets are balanced for amino acids and digestible energy, that there are limited significant differences in growth effects from fish fed any of the plant protein ingredients tested. These findings also provides some support for the effective use of amino acid supplementation to counter any prospective amino acid deficiencies in plant protein meals used in fish diets, as has been shown in other studies (Glencross et al., 2003c).

The difference observed in the tri-iodothyronine levels in fish fed the 40%A treatment, relative to the 0% control treatment, is also consistent with other aberrations noted in the APC product fed at high inclusion levels. Although the tri-iodothyronine levels were also elevated in most other test treatments (including the negative controls), these effects were not significant. The first of a series of three experiments by Burel et al. (1998) also observed changes in the levels of both tri-iodothyronine (T_3) and thyroxine (T_4) in rainbow trout fed diets with *L. albus* kernel

meal included (Burel et al., 1998). Similar to the present study Burel et al. (1998) also noted an increase in the level of T_3 in the plasma/blood in response to lupin inclusion, relative to that of fish fed the fishmeal control diet. However, in contrast to the study by Burel et al. (1998), where they observed a direct negative effect of lupin inclusion on T_4 levels, no significant effects of our treatments on the levels of T_4 were observed in the present study. However, it is acknowledged that this may be limited by the level of variance present in the T_4 data in the present study. Interestingly, in two subsequent experiments by Burel et al. (1998) thyroid hormone effects attributable to the lupin inclusion were not evident. These authors concluded by stating that they believed there was no explicit effect of lupin inclusion on plasma T_3 levels.

The PLRF trial format used in this study presents several experimental advantages to the more typically used experimental designs. Because of the minimisation in feed intake variability between treatments, specific differences between treatments can be more directly related to the diet composition rather than a combination of composition and intake effects. The use of counterpart negative control treatments (in this study the 20%C and 40%C treatments) allows an examination of the discrete value of the test ingredients relative to other reference ingredients, when fed to the fish.

16.4.3 Functional properties of lupin products

The use of rapid viscosity analysis (RVA) techniques in aquaculture nutrition is a relative new advent. Essentially the RVA assessment provides information on the changes of sample viscosity with varying environmental conditions. Typically this technique has been used successfully in assessing wheat starch qualities and diet extrusion parameters (ICC, 1995). Examination of the pasting data generated from the RVA shows the relative hydration of the meal in response to the presence of water and heat (Figure 16.2, A). As the product hydrates it begins to gel reaching a peak viscosity, (Figure 16.2, B). In some products, such as starch, a breakdown of the gel matrix occurs and there is a decrease in the viscosity (Figure 16.2, C). As the RVA sample temperature is cooled towards the end of the analysis the viscosity again increases to a final end viscosity (Figure 16.2, D).

There is further potential in using the RVA for the assessment of feed mix viscosities, which have been shown to affect the digestibility of diets and also the level of endogenous protein loss from the gastrointestinal tract (Simon, 2002). The inclusion of non-starch polysaccharides (NSP) in diets fed to pigs, rats, poultry and fish has been shown to also substantially increase the relative intestine weight (Simon, 2002; Glencross et al., 2004a). It is suggested that increasing the inclusion of NSP also increased the rate of intestinal cell turnover as a consequence of the increase in digesta viscosity (Simon, 2002).

16.4.4 Conclusions

Both of the lupin protein concentrate products evaluated in this study show clear nutritional potential for use in aquaculture diets. Their digestible nutrient and energy value is high, they exhibit few palatability problems and show a discrete nutrient/energy value equivalent to fish meal, soy protein concentrate or *L. luteus* kernel meal. Although there appears clear nutritional value for these products, further technical and economic assessment of their potential is required. Notably, the effects of any processing modifications may also have implications on their subsequent nutritional value and this needs to be accounted for.

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Table 16.1 Nutrient composition of the ingredients used in the studies or comparative discussion (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[b] Pregelled wheat starch	[c] Cellulose	[d] Luteus Kernel Meal	[e] Luteus Protein Conc	[f] Angust Kernel Meal	[e] Angust Protein Conc	[g] Soy Protein Conc	[h] EHC
Dry matter content (g/kg)	917	906	933	903	944	885	942	939	916
Crude protein	770	7	3	547	781	415	690	590	839
Crude fat	68	11	2	87	78	53	93	54	11
Ash	142	3	2	44	37	33	31	79	70
Phosphorus	22	0	0	6	6	4	5	9	9
Organic matter	858	997	998	956	963	967	969	921	930
Gross energy (MJ/kg DM)	21.3	17.2	17.3	20.9	22.2	20.4	22.2	20.3	21.2
Lysine	45.7	0.6	0.0	22.5	31.6	13.9	24.8	28.3	59.5
Threonine	31.9	1.7	0.0	19.9	24.2	16.0	23.0	25.3	39.5
Methionine	21.1	0.0	0.0	4.2	5.1	2.6	4.8	8.5	24.7
Isoleucine	28.4	2.1	0.0	19.5	27.6	15.4	27.0	25.8	46.2
Leucine	54.8	0.0	0.0	44.5	62.9	29.1	50.8	47.7	78.8
Valine	34.0	0.0	0.0	18.5	24.8	14.4	23.3	27.0	61.0
Phenylalanine	29.4	0.0	0.0	21.1	30.4	16.0	27.8	30.2	40.5
Histidine	24.6	0.0	0.0	14.7	18.2	10.3	15.4	15.0	23.7
Arginine	43.2	0.0	0.0	61.1	79.3	47.2	77.9	44.9	33.0

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia. [b] Supplied by Weston BioProducts, Henderson, Western Australia, Australia.

[c] Supplied by ICN Biomedical, Costa Mesa, CA, USA. [d] Supplied by Coorow Seed Cleaners, Coorow, Western Australia, Australia.

[e] Angust Protein Conc.: *L. angustifolius* protein concentrate and Luteus Protein Conc.: *L. luteus* protein concentrate; supplied by Department of Agriculture, South Perth, Western Australia, Australia.

[f] Angust Kernel Meal: *L. angustifolius* kernel meal, supplied by WESFEEDS Pty Ltd, Welshpool, Western Australia, Australia. [g] Supplied by Hamlet-Protein AS, Horsens, Denmark. [h] Supplied by SIGMA, St Louis, Missouri, United States.

Table 16.2 Nutrient and energy digestibilities (%) and total digestible nutrient (g/kg DM) and energy contents of test ingredients (derived from Glencross et al., 2005).

	Fish meal	Luteus Kernel Meal	Luteus Protein Conc	Angust Kernel Meal	Angust Protein Conc	Soy Protein Conc	EHC
Digestibilities							
Energy	99.0	64.2	94.4	53.1	84.2	87.3	91.5
Nitrogen / Protein	87.5	88.6	102.1	85.3	98.4	97.9	92.2
Phosphorus	35.1	183.3	131.5	346.0	138.5	76.3	92.3
Organic Matter	93.1	57.5	92.8	44.6	70.7	67.2	89.1
Digestible Nutrient Levels							
Energy (MJ/kg DM)	21.1	13.4	21.0	10.8	18.7	17.7	19.4
Protein	673	485	781	354	679	578	774
Phosphorus	8	6	6	4	5	7	8
Organic Matter	799	550	893	431	685	619	828

Table 16.3 Formulations of the experimental diets for the palatability trial (all values are g/kg).

		Luteus Protein Conc (LPC).				Angustifolius Protein Conc (APC).					
Ingredient	**Reference**	**10%**	**20%**	**30%**	**40%**	**10%**	**20%**	**30%**	**40%**	**Negative-1**	**Negative-2**
Marker	10	10	10	10	10	10	10	10	10	10	10
CaPO4	0	6	12	18	24	5	10	15	20	0	0
Pre-mix vitamins	3	3	3	3	3	3	3	3	3	3	3
Cellulose	118	108.4	98.8	89.2	79.6	99.4	80.8	62.2	43.6	113	108
Pregelled starch	100	100	100	100	100	100	100	100	100	100	100
Fish oil	168	175	182	189	196	174	180	186	192	168	168
Fish meal	601	492	383	274	165	503.2	405.4	307.6	209.8	601	601
Luteus Protein Conc (LPC)	0	100	200	300	400	0	0	0	0	0	0
Angustifolius Protein Conc (APC)	0	0	0	0	0	100	200	300	400	0	0
DL-Methionine	0	2	4	6	8	3	6	9	12	0	0
L-Lysine	0	1.6	3.2	4.8	6.4	2	4	6	8	0	0
L-Threonine	0	0.6	1.2	1.8	2.4	0.4	0.8	1.2	1.6	0	0
L-Phenylalanine	0	0.6	1.2	1.8	2.4	0	0	0	0	0	0
L-Histidine	0	0.4	0.8	1.2	1.6	0	0	0	0	0	0
L-Valine	0	0.4	0.8	1.2	1.6	0	0	0	0	0	0
Sulfamerazine sodium	0	0	0	0	0	0	0	0	0	5	10

Source of ingredients provided in table 1.

* Supplied by Rhone Poulenc, Goodna, Queensland, Australia. Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g;

Table 16.4 Composition of the experimental diets for the palatability trial (all values are g/kg DM unless otherwise stated).

Ingredient	Reference	Luteus Protein Conc (LPC).				Angustifolius Protein Conc (APC).				Negative-1	Negative-2
		10%	20%	30%	40%	10%	20%	30%	40%		
Dry matter	959	968	970	962	957	972	968	960	958	965	969
Protein	385	380	387	396	405	377	385	391	449	411	421
Fat	239	241	248	245	246	240	247	246	219	233	236
Carbohydrate [a]	280	297	293	291	287	305	293	298	272	261	247
Phosphorus	16	14	14	13	13	14	13	12	11	15	15
Ash	96	82	72	68	63	78	74	66	59	95	96
Gross Energy (MJ/kg DM)	23.5	23.5	23.8	24.3	24.4	23.4	23.9	24.1	23.7	23.4	23.3
Calculated composition											
Lysine	32	29	27	25	23	31	30	29	28	32	32
Threonine	16	15	14	14	13	16	15	15	14	16	16
Methionine	11	10	10	9	8	11	11	11	11	11	11
Isoleucine	18	17	15	14	12	17	16	15	14	18	18
Leucine	30	28	25	23	21	29	27	26	24	30	30
Tryptophan	5	4	4	4	3	5	4	4	4	5	5
Valine	20	19	17	15	14	19	18	16	15	20	20
Phenylalanine	16	15	14	14	13	15	14	14	13	16	16
Histidine	10	10	9	9	9	9	9	9	9	10	10
Arginine	30	30	29	29	28	31	32	33	34	30	30

[a] Carbohydrate content determined based on dry matter – (protein + ash + fat).

Table 16.5 Growth, feed intake and survival of fish fed the experimental diets in the palatability trial (n=4 tanks/treatment).

		Luteus Protein Conc (LPC).				Angustifolius Protein Conc (APC).						Pooled
	Reference	10%	20%	30%	40%	10%	20%	30%	40%	Neg 1	Neg 2	SEM
Initial weight (g/fish)	35.6 [a]	35.6 [a]	35.6 [a]	35.6 [a]	35.6 [a]	35.6 [a]	35.7 [a]	35.5 [a]	35.6 [a]	35.5 [a]	35.8 [a]	0.03
Final weight (g/fish)	126.8 [a]	127.4 [a]	130.5 [a]	130.1 [a]	128.0 [a]	126.4 [a]	123.0 [a]	124.0 [a]	126.4 [a]	117.4 [b]	97.8 [c]	1.41
DGC [a]	4.13 [a]	4.14 [a]	4.25 [a]	4.23 [a]	4.16 [a]	4.12 [a]	4.00 [a]	4.04 [a]	4.11 [a]	3.83 [b]	3.12 [c]	0.048
Gain	91.2 [a]	91.7 [a]	95.0 [a]	94.4 [a]	92.4 [a]	90.8 [a]	87.2 [a]	88.4 [a]	90.8 [a]	81.9 [b]	62.0 [c]	1.42
FCR [b] (g fed : g gain)	0.88 [a]	0.84 [a]	0.82 [a]	0.82 [a]	0.83 [a]	0.83 [a]	0.81 [a]	0.86 [a]	0.83 [a]	0.85 [a]	0.88 [a]	0.005
FCE [c] (g gain : g fed)	1.14 [a]	1.19 [a]	1.22 [a]	1.22 [a]	1.20 [a]	1.20 [a]	1.24 [a]	1.17 [a]	1.20 [a]	1.18 [a]	1.14 [a]	0.005
Food intake (g/fish/d)	1.89 [a]	1.82 [a]	1.83 [a]	1.83 [a]	1.81 [a]	1.78 [a]	1.66 [ab]	1.78 [a]	1.78 [a]	1.64 [b]	1.28 [c]	0.027
Survival (%)	100 [a]	100 [a]	98.7 [a]	100 [a]	100 [a]	98.7 [a]	98.7 [a]	100 [a]	100 [a]	97.5 [a]	100 [a]	0.22
N retention (%)	34.4 [a]	39.1 [ab]	42.2 [b]	41.1 [b]	39.5 [ab]	38.8 [ab]	41.0 [b]	38.0 [ab]	36.5 [a]	35.9 [a]	33.7 [a]	0.52
P retention (%)	28.6 [a]	29.3 [a]	29.4 [a]	37.7 [b]	36.3 [b]	33.7 [ab]	33.4 [ab]	36.4 [b]	38.6 [b]	29.6 [a]	25.9 [a]	0.89
Energy retention (%)	44.0 [a]	46.0 [a]	50.4 [b]	48.8 [ab]	52.2 [b]	48.9 [ab]	49.2 [b]	47.8 [ab]	48.4 [ab]	45.5 [a]	40.0 [a]	0.58

Table 16.6 Formulations of the experimental diets for the protein-limited-restrictively-fed trial (all values are g/kg DM).

Ingredient	0%	20% A	40% A	20% L	40%L	20% C	40% C	20% S	40% S	20% K	40% K	REF
Cr_2O_3 [h]	5	5	5	5	5	5	5	5	5	5	5	5
Ca_2PO_4 [b]	0	12.5	25	14	28	0	0	5	10	5	10	0
Pre-mix vitamins [g]	3	3	3	3	3	3	3	3	3	3	3	3
Cellulose [e]	226	196	166	209.3	192	426	626	171	116	156	86	226
Pregelled wheat starch [f]	100	100	100	100	100	100	100	100	100	100	100	100
Fish oil [a]	156	168.5	181	170.5	185	156	156	160.5	165	153	150	156
L. luteus kernel meal [a]	0	0	0	0	0	0	0	0	0	200	400	0
Soy Protein Conc. [a]	0	0	0	0	0	0	0	200	400	0	0	0
L-Valine [h]	0	0	0	0.8	1.5	0	0	0	0	0	0	0
L-Phenylalanine [h]	0	0	0	1	2	0	0	0	0	0	0	0
L-Histidine [h]	0	0	0	0.5	1	0	0	0	0	0	0	0
Fish meal [a]	510	310.5	111	288	66	310	110	353.5	197	376	242	510
L-Threonine [b]	0	0	0.00	0.10	0.20	0	0	0	0	0	0	0
Angustifolius Protein Conc. [c]	0	200	400	0	0	0	0	0	0	0	0	0
Luteus Protein Conc. [c]	0	0	0	200	400	0	0	0	0	0	0	0
DL-Methionine [b]	0	3.5	7	4	8	0	0	2	4	2	4	0
L-Lysine [b]	0	1	2	3	6	0	0	0	0	0	0	0

Table 16.7 Composition of the experimental diets for the PLRF trial (all values are g/kg DM unless otherwise stated).

	0%	20% A	40% A	20% L	40% L	20% C	40% C	20% S	40% S	20% K	40% K
Dry matter	953	961	958	961	962	965	966	956	948	946	954
Protein	396	394	412	396	407	248	88	408	419	414	435
Fat	233	271	268	251	268	198	193	236	232	242	222
Carbohydrate	241	233	227	251	237	464	665	236	229	222	233
Phosphorus	13	11	9	11	10	8	3	11	10	11	11
Ash	83	63	50	63	50	55	21	75	68	69	64
Gross Energy	22.8	23.3	23.8	23.1	23.7	21.3	20.5	23.0	23.2	23.2	23.2
Calculated composition											
Lysine	27	22	17	22	17	16	6	25	23	23	19
Threonine	14	12	10	12	10	8	3	14	13	12	11
Methionine	9	8	7	8	6	6	2	9	8	8	7
Isoleucine	15	13	11	12	9	9	3	16	16	14	12
Leucine	26	22	19	21	16	16	6	26	26	23	21
Tryptophan	4	4	3	3	2	3	1	4	5	4	3
Valine	17	14	11	14	10	11	4	17	17	15	13
Phenylalanine	13	12	11	12	10	8	3	14	16	12	11
Histidine	8	8	7	7	6	5	2	8	9	8	7
Arginine	26	27	29	24	23	16	6	26	26	26	26

A: *L. angustifolius* Protein Concentrate. L: *L. luteus* Protein Concentrate. C: Cellulose. S: Soybean Protein Concentrate. K: L. *luteus* kernel meal.

Table 16.8 Growth, feed efficiency performance and blood thyroid hormones of fish fed the experimental diets (n=4 tanks/treatment).

	0%	20% A	40% A	20% L	40%L	20% C	40% C	20% S	40% S	20% K	40% K	REF	Pooled SEM
Fish performance criteria													
Initial weight (g)	114.1 [a]	113.3 [a]	114.1 [a]	114.6 [a]	113.9 [a]	113.0 [a]	113.4 [a]	113.8 [a]	113.7 [a]	112.3 [a]	113.6 [a]	114.2 [a]	0.17
Final weight (g)	208.4 [a]	213.2 [a]	202.7 [a]	213.5 [a]	206.5 [a]	170.5 [b]	137.7 [c]	206.9 [a]	199.0 [a]	210.2 [a]	215.5 [a]	253.1 [d]	3.93
DGC [a] (%/d)	2.57 [a]	2.70 [a]	2.44 [a]	2.66 [a]	2.53 [a]	1.69 [b]	0.77 [c]	2.54 [a]	2.37 [a]	2.67 [a]	2.74 [a]	3.50 [d]	0.09
Gain (g/d)	94.2 [a]	99.9 [a]	88.6 [a]	98.9 [a]	92.6 [a]	57.5 [b]	24.4 [c]	93.1 [a]	85.3 [a]	97.9 [a]	101.9 [a]	138.8 [d]	3.88
Survival (%)	100.0 [a]	100.0 [a]	98.3 [a]	100.0 [a]	96.7 [a]	100.0 [a]	98.3 [a]	100.0 [a]	100.0 [a]	100.0 [a]	98.3 [a]	100.0 [a]	0.36
Feed intake (g/fish/d)	2.01 [a]	2.01 [a]	2.04 [a]	2.01 [a]	2.04 [a]	1.98 [a]	1.74 [b]	2.01 [a]	2.01 [a]	2.01 [a]	2.04 [a]	3.13 [c]	0.05
FCR [b] (g fed :g gain)	0.99 [a]	0.92 [a]	1.05 [a]	0.93 [a]	1.01 [a]	1.58 [b]	3.30 [c]	0.99 [a]	1.08 [a]	0.94 [a]	0.91 [a]	1.03 [a]	0.10
FCE [c] (g gain:g fed)	1.01 [a]	1.09 [a]	0.95 [a]	1.07 [a]	0.99 [a]	0.63 [b]	0.30 [c]	1.01 [a]	0.93 [a]	1.06 [a]	1.09 [a]	0.97 [a]	0.10
Blood T_3 (pmol/L)	2.5 [a]	2.2 [a]	4.3 [b]	3.9 [ab]	3.7 [ab]	3.4 [ab]	3.9 [ab]	3.1 [ab]	3.1 [ab]	3.4 [ab]	3.1 [ab]	2.6 [ab]	0.18
Blood T_4 (pmol/L)	0.9 [a]	0.5 [a]	2.0 [a]	1.7 [a]	1.5 [a]	2.1 [a]	1.1 [a]	2.0 [a]	0.7 [a]	0.9 [a]	1.4 [a]	0.9 [a]	0.16

[a] Daily Growth Coefficient.

[b] Food Conversion Ratio; grams of dry matter consumed per grams live-weight gain.

[c] Food Conversion Efficiency; grams live-weight gain per dry matter food consumed.

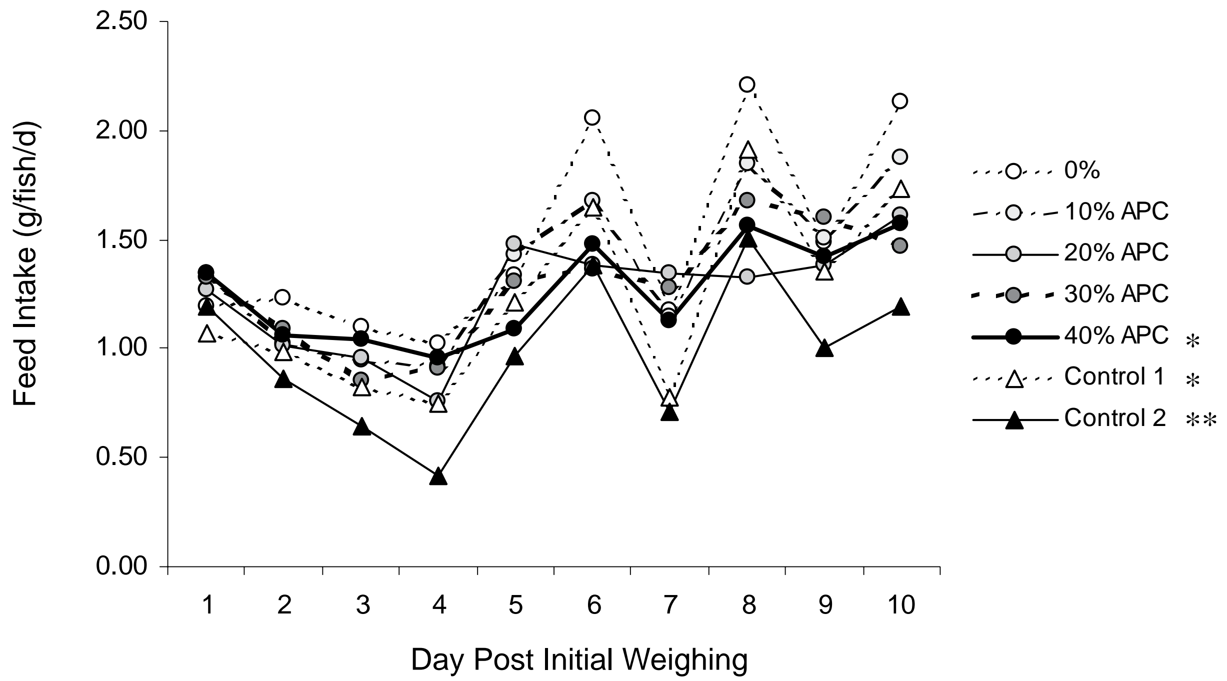

Figure 16.1 Variability in daily feed intake of the *L. angustifolius* Protein Concentrate (APC) series of treatments by rainbow trout over the first ten days of the experiment (n=4 tanks/ treatment). Treatments marked (* or **) are significantly different from the 0% reference at P < 0.05 or P < 0.01.

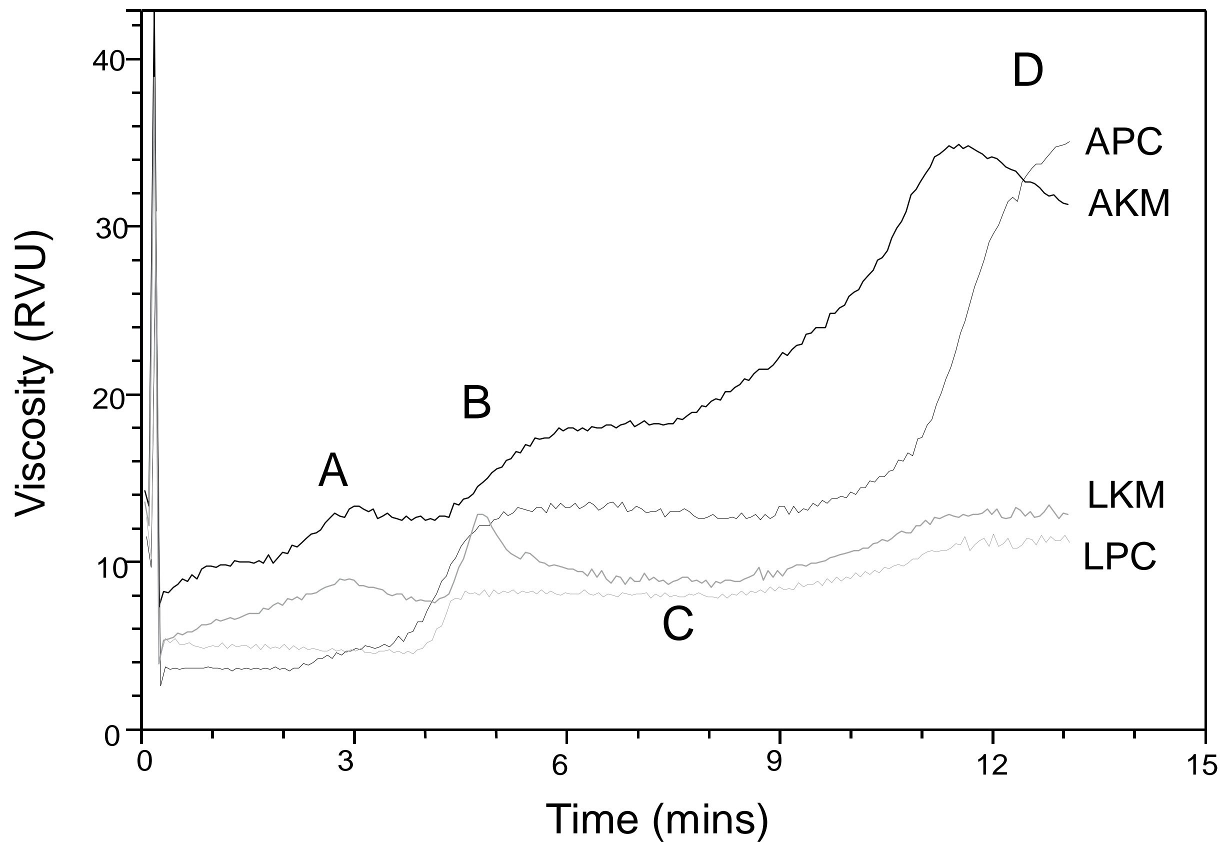

Figure 16.2 Rapid-Visco-Analyser (RVA) pasting curve characteristics of key test ingredients.

17.0 Evaluation of the influence of drying process on the nutritional value of lupin protein concentrates when fed to rainbow trout (*Oncorhynchus mykiss*)[a]

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Abstract

A series of studies were undertaken to examine the effect of drying processes on the composition, digestibility and utilisation efficiencies of different types of lupin (*L. angustifolius* cv. Myallie) protein concentrates when fed to rainbow trout. Three different LPC drying methods (freeze-drying: FD, spray-drying: SD, and heat-drying: HD) were studied. Significant effects of drying process were observed on the composition of the LPC; most notable was the relative increase in the level of crude fibre and decrease in crude protein with the heat-dried product. The digestibilities of each of the LPC were assessed using the diet-substitution method with faecal collection undertaken using stripping techniques. No significant differences in the digestibilities of protein or energy, or total digestible protein and energy concentrations were observed among the LPC. To assess the utilisation of protein and energy, fish were fed diets with a 300 g/kg inclusion level of either the spray-dried or heat-dried LPC. A third fishmeal based reference diet was also used. The diets were formulated to equivalent digestible protein and energy specifications based on predetermined digestibility values. Each of the diets was fed at one of three ration levels and an additional starved treatment was also included. In a 28-day growth study, fish of 96.4 ± 1.7 g (mean ± S.D.) kept in freshwater at 13.9 ± 0.2°C grew in accordance with their ration level, but with some significant differences observed among the diets. This experiment shows that the dietary inclusion of the heat-dried LPC significantly reduced the efficiency of energy gain. Utilisation of digestible protein at lower digestible protein intake levels did not appear less efficient with the heat-dried LPC, but at higher protein intake levels it was not as efficiently used as spray-dried LPC or fishmeal protein. A greater proportion of the nitrogen excretion from the fish fed the heat-dried LPC diet was observed as urea. This study demonstrates that the drying regime used on a processed grain product may not affect the ability of fish to digest the protein and energy from that grain product, but may affect the ability of the fish to utilise the dietary digestible protein and energy of the ingredient.

[a] Published as: Glencross, B.D., Hawkins, W.E., Evans, D., McCafferty, P., Dods, K., and Sipsas, S. 2007. Heat damage during some drying techniques affects nutrient utilisation, but not digestibility of lupin protein concentrates fed to rainbow trout (*Oncorhynchus mykiss*). Aquaculture 265, 218 – 229.

17.1 Introduction

Lupin (*Lupinus* spp.) meals are one ingredient that have been used to reduce reliance on fish meal as the primary protein source n aquaculture diets. Typically it is the kernel meals of lupins that are being used in these diets (Burel et al., 2000; Farhangi and Carter, 2001; Glencross and Hawkins, 2004; Glencross et al., 2004a). However, like many plant protein meals there are limitations to the inclusion level of most varieties of lupins in fish diets, often as a consequence of their inherent protein level not being sufficiently high enough to justify higher inclusion levels. It would be of substantial value if they had slightly enhanced nutritional characteristics, such as higher protein levels and lower non-starch polysaccharide (NSP) levels (Hardy, 1996).

Like many plant protein meals there are also prospective anti-nutritional factors (ANF) in lupin kernel meals (Francis et al., 2001; Glencross et al 2003; Glencross et al., 2006b). To improve the potential value of lupin meals the development of a series of prototype protein concentrates has progressed and a range of products of varying compositional characteristics have been produced and evaluated (Glencross *et al*., 2004a; 2005; 2006a). To further develop the commercial potential for these products it was identified that developing cost-effective drying techniques that did not reduce the nutritional value of the product, would be critical to the viability of the product (Dale, 1996; Kingwell, 2003).

A range of drying processes are used, where necessary, to produce both plant and animal protein meals. Among these drying processes, freeze-drying is considered one of the least damaging and is routinely used as a laboratory preparation method for this reason (Petterson et al., 1999). On the other hand, oven drying is well known as being relatively destructive (van Barneveld et al., 1994a; 1994b; Glencross et al., 2004d). This is particularly so with plant meals, where chemical reactions can significantly reduce the nutritional value of the protein content of the meal through the occurrence of condensation reactions between lysine residues and free-sugars in the meal (Ford and Shorrock, 1971; Erbersdobler, 1977). This reaction is usually referred to as a Maillard reaction (Oste, 1984). Commercial drying processes such as spray-drying and ring-drying are routinely used to dry protein meals such as blood meal, soy isolates and milk proteins (Fellows, 2000). The impact of heat on the nutritional value of a range of raw materials to a range of monogastric species has been reported (van Barneveld et al., 1994a; Bureau et al., 1999; Medel et al., 2004; Peres et a., 2003; Glencross et al., 2004d). Of these studies, most have reported some changes in digestible nutrient and energy value (Bureau et al., 2000; Peres et a., 2003; Glencross et al., 2004d). Few studies have examined the impact of variations or lack there-of on nutrient and energy availability from heat-treated raw materials. Work with pigs has shown that digestible value and available value are not always directly related (van Barneveld et al., 2004b). This study reports on the nutritional evaluation of several drying processes used to produce protein concentrates from *L. angustifolius*, when fed to Rainbow trout, *Oncorhynchus mykiss*.

17.2 Methods

In the present study two separate experiments were undertaken to evaluate the effects of drying regime on the nutritional value of three lupin protein concentrates. Firstly an ingredient digestibility evaluation was undertaken to measure the digestible protein and digestible energy value of each protein concentrate. Following the digestibility experiment, a second experiment was designed to examine the protein and energy utilisation efficiencies associated with diets where a 300 g/kg amount of each protein concentrate was included. Diets in the utilisation study

were formulated to be iso-proteic and iso-energetic on a digestible basis, based on the outcomes from experiment 1. The objective of experiment 2 being to ascertain whether the protein and/or energy from the protein concentrates was used any less efficiently than that of the fishmeal protein and energy of the reference diet. The specifics of each study and some general methods used are detailed subsequently.

17.2.1 General methods

17.2.1.1 Ingredients and ingredient preparation

Composition and source of all of the ingredients used is presented in Table 17.1. Lupin kernel meal (*Lupinus angustifolius,* cv. Myallie) was obtained from a commercial grain miller and ground to < 600μm particle size. To make the protein concentrates, the kernel meal was solubilised in water and the pH adjusted to 9.0 with NaOH (2.0 M) with vigorous stirring for 60 min. After mixing, the solution was filtered through a 500 μm filter bag to separate the non-solubilised material from the solubilised protein. The protein solution was then brought to a pH of 4.0 with the addition of HCl (2.0 M) to precipitate out the solubilised protein. The protein precipitate was decanted and dried in a freeze drier, spray-drier or oven-dried at 150°C for 12 h. The extraction processes are based on those reported in Lasztity et al. (2001). Following the drying processes, the LPC was re-milled to ensure all particles were < 800 μm particle size. The remaining feed ingredients were obtained as detailed in Table 17.1.

17.2.1.2 Chemical analysis

Respective samples of ingredients, diet, faecal and whole-body samples were analysed for a variety of analytes, depending on experiment, including dry matter, ytterbium, ash, fat, nitrogen, phosphorus and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Ytterbium and phosphorus levels were determined using Inductively Coupled Plasma – Atomic Emission Spectroscopy (ICP-AES) (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Kjeldhal digestion, based on N x 6.25. Crude fat content was determined gravimetrically following extraction of the lipids according to the Soxhlet method (AOAC International, 2005). Crude fibre was determined based on loss of residue on ignition at 550°C following hydrolysis of a sample in H_2SO_4 and NaOH. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. All chemical analyses were undertaken by professional analytical chemists (Chemistry Centre, Perth, WA, Australia).

Total water ammonia concentrations were determined from thawed water samples using a Hach ammonia test kit and laboratory spectrophotometer. The urea concentration was determined based on the concentration of ammonia following the conversion of urea to ammonia using an enzyme preparation of urease. Water samples were incubated at 25°C with 1.0 g/L of urease (SIGMA, St Louis, Missouri, United States) until no further increase in the amount of liberated ammonia was determined. Samples were compared against both blanks and standards.

17.2.2 Ingredient digestibility

17.2.2.1 Diet preparation

A basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 160 g/kg DM fat and an inert marker (ytterbium oxide 1 g/kg) (Table 17.2). A basal mash was prepared

and thoroughly mixed, forming the basis for all diets in this experiment. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash, (see Table 17.2). Diets were then processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 24 h before being allowed to cool to ambient temperature in the oven. A basal diet was prepared in a similar manner, but without the addition of any test ingredient.

17.2.2.2 Fish management – Experiment 1

Hatchery-reared Rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 L). Freshwater (salinity < 1 PSU) of 16.0 ± 0.1°C at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 10 trout of 442 ± 58 g (mean ± S.D.), with four replicates per treatment.

Fish were hand fed the diets daily to apparent satiety as determined over three separate feeding events between 1600 and 1800hrs. The trout were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced (Wybourne and Carter, 1999). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). Fish were netted from their respective tank, placed in a smaller aerated tank containing an anaesthetic (0.002 mL/L as active compound isoeugenol) until they lost consciousness. The faeces were then removed from the distal intestine using gentle abdominal pressure. Care was maintained to ensure that the faeces were not contaminated by urine or mucous. After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and later stored in a freezer at -20°C. Hands were rinsed in freshwater after each fish. Stripped faeces were collected during 0800 to 1000hrs over a four-day period, with each fish only stripped twice during this period and not on successive days.

17.2.2.3 Digestibility analysis

Differences in the ratios of each parameter relative to ytterbium content in the feed and faeces in each treatment, were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional variables examined in each diet based on the following formula (Maynard and Loosli, 1969):

$$ADC_{diet} = 1 - \left(\frac{Yb_{diet} \times Nutrient_{faeces}}{Yb_{faeces} \times Nutrient_{diet}} \right)$$

where Yb_{diet} and Yb_{faeces} represent the ytterbium content of the diet and faeces respectively, and $Nutrient_{diet}$ and $Nutrient_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$

and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998).

17.2.3 Protein and energy utilisation

17.2.3.1 Diet development

All experiment diets were formulated to be iso-proteic (400 g/kg) and iso-energetic (18.0 MJ/kg) on a digestible protein/energy basis. Digestibility coefficient values for key ingredients were based on those reported earlier (Glencross et al., 2005) and from this study and used the same batches of ingredients in each case. Diets were processed by the addition of water (about 30% of mash dry weight) to all ingredients while mixing to form a dough, which was subsequently screw-pressed through a 4 mm diameter die using a pasta maker. The resultant moist pellets were then oven dried at 70°C for approximately 24 h before being air-cooled, bagged and stored at -20°C. Formulations and proximate composition for all diets are presented in Table 17.4 and 17.5 respectively.

17.2.3.2 Fish management – Experiment 2

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 L). Freshwater (salinity < 1 PSU; Dissolved oxygen 9.6 ± 0.5 mg/L, mean ± S.D.) of 13.9 ± 0.2°C (mean ± S.D.) at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 20 trout of 96.4 ± 1.7 g (mean ± S.D.; n = 240). Photoperiod was maintained at 12:12 (light:dark). Treatments were randomly assigned amongst 30 tanks, with each treatment having three replicates. For all weight assessments the fish were netted from their respective tank, placed in a smaller aerated tank containing isoeugenol (0.002 mL/L) until they lost consciousness.

The fish were fed at one of four levels of feed intake ranging from a starved treatment to apparent satiety and two intermediary feed levels, once daily at 0800, for 28 days. Apparent satiety was determined by a loss in feeding activity, which was reached after three feeding sessions over a one-hour period. Any uneaten feed was removed from each tank one hour later and the uneaten portion dried and weighed to allow the determination of daily feed intake based on correction factors for leaching losses sustained over an equivalent period (Helland et al., 1996).

Fish were individually re-weighed after four weeks, with all fish within each tank used to determine the average weight gain/loss per tank and treatment (Table 15.5). Five fish were taken as an initial sample for composition analysis. At the end of the study three fish were taken from each tank for whole body composition analysis. Growth was assessed as mean weight gain and daily growth coefficient (DGC). DGC was calculated as (Kaushik, 1998):

$$DGC = \frac{\left(W_f^{1/3} - W_i^{1/3}\right)}{t} \times 100$$

Water samples were collected from the starved and satiety fed treatments to determine the proportion of nitrogenous waste excreted as either ammonia or urea. Water samples were collected at six hours post-feeding and kept frozen prior to analysis.

17.2.3.3 Digestibility analysis

Digestibilities of protein and energy were determined at the end of the growth study from each of the test and control diets. Faeces were collected using stripping techniques based on those reported earlier. Calculation of diet digestibility parameters was the same as detailed in section 17.2.2.3.

17.2.3.4 Protein and energy retention

Protein (N) and Energy (E) retention were determined based on the mass gain in both N and E over the course of the experiment, against the respective consumption of N and E. This was determined on both a digestible and gross basis (Table 17.5 and Figures 17.1 and 17.2). Both values were calculated according to the following formula (Maynard and Loosli, 1969):

$$Nitrogen\ Retention = \left(\frac{Nt - Ni}{Nc}\right) \times 100$$

Where *Nt* is the nitrogen content of the fish in a specific replicate at time *t* and *Ni* is the initial nitrogen content of the fish from the beginning of the study (n=3 replicates of 3 representative fish). *Nc* is the amount of nitrogen consumed by the fish from the time of initial assessment to time t. Determination of Energy retention was achieved the same way, but with the substitution of the relevant energy criteria where the corresponding nitrogen criteria are indicated in the equation. In this study these values are determined based on gross nitrogen and energy intake only.

To provide some independence of size effects, modelling of the protein and energy retention efficiency data was done with respect to known energy and protein body-weight exponents for rainbow trout of $x^{0.8}$ and $x^{0.7}$ respectively (Cho and Kaushik, 1985).

17.2.4 Statistical analysis

All values are mean ± SE unless otherwise specified. Effects of diets were examined by ANOVA using the software package Statistica (Statsoft®, Tulsa, OA, USA). Feed intake levels and diet effects were examined by MANOVA using the software package Statistica. Levels of significance were determined using an LSD test, with critical limits being set at $P < 0.05$. Linear and non-linear regression was undertaken using Microsoft Excel.

17.3 Results

17.3.1 Ingredient composition

Minor changes in the composition of the LPC were observed among the different drying techniques. Dry matter was highest in the heat-dried LPC and lowest in the freeze-dried LPC (Table 17.1). Protein was also slightly elevated in the heat-dried LPC with the freeze-dried LPC also the lowest in protein. Crude fat was lowest in the heat-dried LPC and highest in the freeze-dried LPC. Crude fibre was highest in the heat-dried LPC and lowest in the freeze-dried LPC (Table 17.1).

17.3.2 Ingredient and diet digestibility

In experiment one, no significant differences between the digestible protein and energy value of the protein concentrates produced using heat, spray or freeze –drying were noted (Table 17.3). Digestibility of energy was significantly higher in the protein concentrates compared to the lupin kernel meal (MKM), but not the enzymatically-hydrolysed casein (EHC). Protein digestibility for the protein concentrates was high and in some cases significantly higher than that of the EHC.

In experiment two, a lower digestible protein and energy level was measured from the H-diet. A higher digestible energy value was also measured from the S-diet (Table 17.4).

17.3.3 Fish growth and feed utilisation

Growth, as measured by weight gain, of fish in each treatment positively responded to increased ration levels (Table 17.5). Fish in the satietal fed component of the H-diet treatment did not gain as much weigh as fish from corresponding satietal fed components within the R-diet and S-diet treatments. At reduced ration levels growth of fish was marginally, but not significantly less in the H-diet fed fish. There were no differences between the growth of fish fed the R and S diets.

Significant differences in apparent satietal feed intake were observed between the H-diet (heat dried PC) and the R (reference/fish meal) and S (spray-dried PC) diets. There were no significant differences in feed intake between the R and S diets.

Feed conversion ratios (FCR) varied between diets and ration levels. Within treatments, the highest (poorest) FCR were observed at the lowest fed ration level. This was consistent for all treatments. Among treatments, FCR were generally higher (poorer) for the H-diet fed fish, when pair-wise comparisons were made amongst treatment ration levels.

17.3.4 Energy utilisation

Efficiency of energy utilisation over lower digestible energy intake levels was linear, but over the full range was better described by a curvilinear function. The quadratic equations for each relationship are given as equations 1, 2 and 3. Significant differences between the diets were observed with respect the utilisation of dietary digestible energy (Figure 17.1). Energy utilisation efficiency was significantly lower for the fish fed the heat- dried LPC (H) diet (Equation 2). No significant differences in energy utilisation efficiency were observed between the reference (R: equation 1) and the spray-dried LPC (S: equation 3). Over the lower linear range the energy utilisation efficiency of the fish fed the R and S diets was described by the linear equation of; $y = 0.954x - 38.229$, $R^2 = 0.993$. Over the lower linear range the energy utilisation efficiency of the fish fed the H diet was described by the linear equation of; $y = 0.843x - 38.362$, $R^2 = 0.943$. Significant differences among the diets in the energy utilisation efficiency were determined over this data range at $P < 0.1$, but not at $P<0.05$. Over the full data range and for all treatments the energy utilisation efficiency was described by the linear equation of: $y = 0.763x - 30.051$, $R^2 = 0.974$.

Equation 1.

$$\text{Energy gain (Diet R)} = -0.001*(\text{DE intake})^2 + 1.118*(\text{DE intake}) - 40.297,\ R^2 = 0.996$$

Equation 2.

$$\text{Energy gain (Diet H)} = -0.001*(\text{DE intake})^2 + 1.005*(\text{DE intake}) - 40.599,\ R^2 = 0.972$$

Equation 3.

$$\text{Energy gain (Diet S)} = -0.001*(\text{DE intake})^2 + 1.091*(\text{DE intake}) - 40.765,\ R^2 = 0.\ 0.998$$

17.3.5 Protein utilisation

Efficiency of protein utilisation over lower digestible protein intake levels was linear, but over the full range of digestible protein intake in this study, was better described by a curvilinear

function. The quadratic equations for each relationship are given as equations 4, 5 and 6. Significant differences between the diets were observed with respect to the utilisation of dietary digestible protein (Figure 17.2). Protein utilisation efficiency was significantly lower for the fish fed the heat- dried LPC (H) diet (Equation 5). Although the utilisation of protein by fish fed the S diet was lower than that of the R diet, no significant differences in protein utilisation efficiency were observed between the two treatments (R: equation 4) and the spray-dried LPC (S: equation 6). Over the linear region of lower digestible protein intakes there were no significant differences in the protein utilisation efficiency among the fish fed any of the three diets. Over this lower linear range the protein utilisation efficiency of the fish fed all three diets was described by the linear equation of; $y = 0.609x - 0.208$, $R^2 = 0.956$.

Equation 4.

$$\text{Protein gain (Diet R)} = -0.043*(\text{DP intake})^2 + 0.730*(\text{DP intake}) - 0.216,\ R^2 = 0.988$$

Equation 5.

$$\text{Protein gain (Diet H)} = -0.094*(\text{DP intake})^2 + 0.824*(\text{DP intake}) - 0.274,\ R^2 = 0.974$$

Equation 6.

$$\text{Protein gain (Diet S)} = -0.037*(\text{DP intake})^2 + 0.641*(\text{DP intake}) - 0.214,\ R^2 = 0.983$$

17.3.6 Nitrogen excretion

Nitrogen excretion differed significantly among the three treatments. A greater proportion of nitrogen was excreted as urea in the H-diet fed fish than that excreted from the R-diet and S-diet fed fish. The higher level of nitrogen excreted as urea was also noted of fish from the starved treatment. There were no differences in nitrogen excretion patterns between the R-diet and S-diet fed fish.

17.4 Discussion

This study examines two aspects of the dry process on the nutritional value of lupin protein concentrates (LPC). Initially each LPC is evaluated for the digestible protein and energy value, followed by a second experiment where a comparison of the utilisation efficiencies of key nutrients and energy from diets with inclusion of one of the three LPC's, provides sound evidence of the nutritional impact of heat drying on an ingredient when fed to a salmonid. The effects seen show that the inclusion of a heat-dried product has a negative impact on key nutrient or energy utilisation by this animal. However the work also shows that the negative impacts of ingredient processing may not necessarily be apparent when assessed through digestibility studies.

The application of heat to the LPC used in this study also influenced their nutrient composition. This observation is also consistent with those observations reported on heat-treated field pea (*Pisum sativum* cv. Dundale) by van Barneveld (1994a). One of the key functional observations noted of the changes in the nutrient composition of the LPC was the marked increase in crude fibre. This was also consistent with the observations of van Barneveld (1994a). Van Barneveld also noted a much greater increase in neutral detergent fibre extract, though this was not assessed in the present study. These changes in fibre levels in the raw materials provide an important insight into the nutritional changes that occur in vegetable meals when exposed to heat.

The results of the digestibility assessment in the first experiment of the present study showed that the component ingredients of heat, spray or freeze –dried LPCs were similar in their levels

of protein and energy digestibility. This result is similar to the observations of van Barneveld (1994a), who noted no significant deleterious effects of heat on ileal digestibility of nitrogen and amino acids of heat-treated peas when fed to pigs. The result however contrasts work on heat-treated canola meals, which showed a marked reduction in protein and energy digestibility of the heat-dried meals when fed to red seabream, *Pagrus auratus* (Glencross et al., 2004d). Bureau et al. (2000) also noted significant differences among blood meals dried using different methods. In that study the spray-dried meals had the highest digestibilities and the heat-dried products a significantly lower protein and energy digestibility. It is suggested that the composition of the raw material plays an important role in the effect heat has on the digestible nutrient and energy value of such meals. Notably, the type of proteins and the type of carbohydrate classes present may prove to be the difference between no effect or a marked effect of heat on the digestibility of the individual ingredients.

Interestingly, despite all three diets in the second experiment being formulated to provide the same digestible protein and energy characteristics, a significantly lower digestible energy and protein content of the heat-dried LPC (H) diet was measured. In contrast a higher level of digestible energy was measured from the fish fed the spray-dried (S) LPC diet. These observations provide some indication of the non-additive effects of formulating with grain protein meals, in this case a negative effect for the H-diet and a positive effect for the S-diet. Reasons for this discrepancy are suggested to be related to the change in levels of fibre present in the LPC and in particular possible high levels of neutral detergent fibre (van Barneveld, 1994a).

The heat-dried LPC diet (H) was clearly less efficiently utilised than the other two diets, with less growth and higher FCR values observed between the H-diet and the other treatments. The fish fed the S-diet grew well, but at satietal feeding levels the growth was not as high as that of the reference (R) diet. This difference was not significant though. It is suggested that the high inclusion level (300 g/kg) of the LPC in this experiment may have introduced a methionine limitation.

Some significant effects were also observed on the influence of heat- drying on feed intake (palatability). A reduction in the satietal feed intake was observed of the H-diet (heat dried PC) compared to the R (reference/fish meal) and S (spray-dried PC) diets. A similar reduction in feed intake was observed in a study on heat-dried canola meals fed to *P. auratus* (Glencross et al., 2004d). Both of these effects being counter to the known effects of increased satietal intake of feeds of lower digestible energy value (Kaushik and Medale, 1998). It is suspected that the heat applied to grain meals in these studies is in effect "caramelising" some of the sugars and that this effect is possibly reducing the palatability of these raw materials to rainbow trout. In a study by Peres et al. (2003), the application of heat (130°C at 10, 20, 30 and 40 minutes) to defatted ray-soybean meal was shown to increase the protein digestibility of the raw material although it decreased the protein dispersability index of the raw material. This may be directly related to the reduction in anti-protease activity of trypsin inhibitor in the soybean meal with the increasing level of heat treatment. However, regression analysis supports that there is an increase in feed intake by fish fed the soybean meal with progressively longer heat application. This suggests that there has actually been a reduction in digestible energy value of the soybean meal or that there has been the destruction of some anti-palatability factor. In contrast, van Barneveld et al's. (1994a; b; c; d) studies with pigs showed a minor increase in feed intake of the hottest heat-treatment (165°C) on field peas. However, as pointed out by van Barneveld et al. (1994d), it is not possible to draw general conclusions from one protein concentrate to another.

The lower efficiency of energy and protein utilisation of the heat- dried LPC by fish fed the H-diet demonstrates that there has been a reduction in the availability of energy and protein to the fish from this diet and therefore the heat-dried LPC. That both the energy and protein utilisation of the spray-dried LPC was not reduced is consistent with other studies that have shown that provided there are is no reduction in availability, and that provided that data is examined on an equivalent digestible basis, that plant protein meals can be used as efficiently as animal protein meals as an energy source (Glencross et al., 2006a).

This reduction in energy utilisation efficiency is supported by the data on nitrogen excretion. This data shows that there is a higher proportion of urea excreted by the fish indicating a reduction in the efficiency of protein metabolism by the animals. It is plausible that condensation reactions among amino-acid residues and remnant carbohydrates in the LPC may have produced amino acids conjugated to sugar molecules (Hurrell and Carpenter, 1977; Erbersdobler, 1977). This has possibly reduced the ability of the fish to metabolise the amino acids and thereby increase their excretion of incompletely metabolise nitrogenous products (urea) and a reduction in the overall energy gained from the feed (Ford and Shorrock, 1971).

The marginal departure from linearity observed in the present study of the relationship between energy gain and energy intake, particularly at the upper levels of energy intake, contrasts much of that reported by other workers (Azevedo et al., 1998; Rodehutscord and Pfeffer, 1999). However, the feed intake levels and growth achieved are much greater in the present study and this difference is likely to be a contributing factor to this effect.

To allow a comparison of the observed effects against other published works, the data was further reviewed on linear regression basis across the full range of digestible energy intakes. On this basis, the efficiency of energy utilisation differed marginally among each of the treatments. For all treatments combined an efficiency of $k_E = 0.76$ was determined. This comprised of values that ranged from $k_E = 0.78$ for the R-diet to $k_E = 0.72$ for the H-diet. These energy efficiencies are higher than that observed in many other studies on rainbow trout, where the utilisation of energy for energy gain (k_E) was 0.61 regardless of feeding level as well as temperature (Azevedo et al., 1998) or $k_E = 0.68$ in another study (Rodehutscord and Pfeffer, 1999), but is consistent with other studies ($k_E = 0.74$) with this particular strain of rainbow trout (Glencross et al., 2002; Molony et al., 2004).

Utilisation of dietary protein by the fish in the present study differs from that of other studies in that the relationship between protein intake and protein gain is curvilinear, whereas in other studies it has been linear over the full range studied (Lupatsch et al., 2001). The primary feature of the relationship in the present study that might explain this difference in linearity is that in the present study the feed intake and therefore protein intake by the fish is substantially higher, with a deterioration in efficiency only seen above a protein intake of 2 g/ $kg^{0.7}$ /d. In the lower linear range of the relationship, the determined protein utilisation efficiency of 0.61 from the present study is marginally higher than the value of 0.52 reported by Lupatsch et al. (2001) for *Dicentrarchus labrax*. This higher protein efficiency value is consistent with the higher energy utilisation efficiency of this strain of rainbow trout. There were no significant differences in protein utilisation efficiency among the treatments over the lower protein intake levels.

Significant differences were however seen in the quadratic regression of the protein utilisation efficiency between the H-diet and the other two diets. Differences were noted primarily in the degree of curvature not the gradient of the quadratic function. This plateauing of performance is typical of a limitation in dietary energy with a certain degree of feed intake. It is suggested that the lower protein utilisation efficiency of the H-diet at the higher ration levels is consistent with

a reduction in energy generation from assimilated amino acids from this diet. This would also be consistent with the observations in the differences in ammonia and urea excretion among the treatments. The protein utilisation of fish fed the S-diet while not significantly lower than that of the reference (R) diet was marginally lower at the highest intake level. It is suggested that this may be attributable to potential marginal methionine limitation. The difference between the S-diet and the H-diet therefore being the specific effect attributable to heat-drying of the LPC.

This study also shows the limitations of a using a simple satietal feeding strategy in assessing diets (and by inference raw materials), as such strategies tend to be confounded by feed intake variability. The present strategy shows a more robust approach to determining the nutritional value of a diet, independent of intake effects, by examining a regression approach of growth response at a range of ration levels.

The results from this study show that even though the application of heat may have no effect on the digestible nutrient and energy value of the raw materials, their nutritional value can still be significantly impaired. Even when the diets are formulated on a digestible nutrient and energy basis, the interaction of certain components can still affect the resultant digestible value of the diet. Despite these discrepancies in diet digestibilities, it was also observed that even when the diets are compared on an equal digestible protein and energy basis, that heat-dried LPC was not used as efficiently for growth, with reductions in both the efficiency of energy utilisation and the use of protein from this raw material. These observations show that care needs to be taken in the application of heat-treated raw materials to fish diets and due regard given to the differences between the digestibilities and availabilities of nutrients and energy in the diets.

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Table 17.1 Nutrient composition of the ingredients used in the studies (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[b] Pregelled wheat starch	[c] Cellulose	[d] LPC Oven Dried	[d] LPC Spray Dried	[d] LPC Freeze Dried	[e] Lupin Kernel Meal	[c,f] EHC
Dry matter content (g/kg)	917	906	933	961	943	932	908	916
Crude protein	770	7	3	871	862	744	466	839
Crude fat	68	11	2	44	83	130	83	11
Ash	142	3	2	77	49	30	34	70
Phosphorus	22	0	0	8	8	7	6	9
Crude Fibre	11	2	762	11	6	1	37	2
Gross energy (MJ/kg DM)	21.3	17.2	17.3	26.2	25.0	24.8	20.7	21.2
Arginine	43	0	0	74	79	77	42	33
Histidine	25	0	0	12	14	15	9	24
Isoleucine	28	2	0	26	30	28	15	46
Leucine	55	0	0	1	0	0	26	79
Lysine	46	1	0	19	19	19	11	60
Methionine	21	0	0	5	6	5	2	25
Phenylalanine	29	0	0	26	30	28	14	41
Threonine	32	2	0	21	23	23	14	40
Valine	34	0	0	23	26	24	14	61

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia.

[b] Supplied by Weston BioProducts, Henderson, Western Australia, Australia.

[c] Supplied by ICN Biomedical, Costa Mesa, CA, USA.

[d] LPC: *L. angustifolius* (cv Myallie) Protein Concentrate, Supplied by Department of Agriculture, South Perth, Western Australia, Australia.

[e] *L. angustifolius* (cv Myallie) kernel meal, Supplied by Coorow Seed Cleaners, Coorow, Western Australia, Australia.

[f] EHC: Enzymatically-hydrolysed casein.

Table 17.2 Formulations of the experimental diets for the digestibility trial (all values are g/kg).

Ingredient	Basal	LPC-FD	LPC-OD	LPC-SD	MKM	EHC
Fishmeal [a]	650	455	455	455	455	455
Fish oil [a]	110	77	77	77	77	77
L. angustifolius - LPC (Freeze dried)	0	300	-	-	-	-
L. angustifolius - LPC (Heat dried)	0	-	300	-	-	-
L. angustifolius - LPC (Spray dried)	0	-	-	300	-	-
L. angustifolius KM (Myallie)	0	-	-	-	300	-
E H Casein	0	-	-	-	-	300
Pregelled wheat starch	150	105	105	105	105	105
Cellulose	79.0	55.3	55.3	55.3	55.3	55.3
Vitamin and mineral premix [b]	10	7	7	7	7	7
Ytterbium oxide [c]	1.0	0.7	0.7	0.7	0.7	0.7

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia.

[b] Supplied by Rhone Poulenc, Goodna, Queensland, Australia.

[c] Supplied by SIGMA, St Louis, Missouri, United States.

Table 17.3 Nutrient and energy digestibilities (%) and total digestible nutrient (g/kg DM) and energy contents of test ingredients.

	LPC-FD	LPC-OD	LPC-SD	MKM	EHC	Pooled SEM
Digestibilities (%)						
Energy	92.7 [a]	96.9 [a]	91.6 [a]	76.2 [b]	94.4 [a]	2.9
Nitrogen / Protein	96.5 [ab]	103.8 [a]	95.9 [ab]	102.0 [a]	91.8 [b]	1.9
Digestible Nutrient Levels						
Energy (MJ/kg DM)	23.0	23.2	22.9	15.6	20.7	
Protein (g/kg DM)	718	734	733	417	782	

Different superscripts indicated significant ($P < 0.05$) differences among treatments.

Table 17.4 Formulations and composition of the experimental diets for the growth and palatability trial (all values are g/kg).

Ingredient	Reference (R)	Heat-Dried (H)	Spray-Dried (S)
Ytterbium oxide	1	1	1
Pre-mix vitamins**	5	5	5
Cellulose	151	202	185
Pregelled starch	50	50	50
Fish oil	144	169	167
Fish meal	649	273	292
MPC-Spray dried	0	0	300
MPC-Oven dried	0	300	0
Composition as Determined (g/kg DM)			
Dry matter content (g/kg)	952	953	956
Crude protein	507	471	501
Digestible protein *	434 ± 0.9^{a}	361 ± 6.5^{b}	455 ± 0.4^{c}
Crude fat	210	216	218
Ash	109	51	56
Phosphorus	20	11	12
Crude fibre	82	154	131
Gross energy (MJ/kg DM)	23.6	24.7	24.9
Digestible energy (MJ/kg DM)*	17.6 ± 0.23^{a}	17.0 ± 0.44^{b}	18.5 ± 0.12^{c}
Arginine	32	29	30
Histidine	11	8	8
Isoleucine	19	14	14
Leucine	32	23	24
Lysine	34	21	22
Methionine	12	6	7
Phenylalanine	17	12	13
Threonine	17	12	12
Tryptophan	5	4	4
Valine	22	14	15

Different superscripts indicated significant ($P < 0.05$) differences among treatments.

* determined from faeces collected using faecal stripping at the end of the growth trial from the satietal fed fish in each treatment.

**Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g;

Table 17.5 Growth performance, composition and nutrient retention of fish fed the experimental diets.

	Starved	Reference			Heat-Dried			Spray-Dried			Pooled
		L	M	H	L	M	H	L	M	H	SEM
Fish performance criteria											
Initial weight (g)	96.0	96.0	97.7	94.1	98.2	97.9	96.0	96.5	95.7	96.2	0.25
Final weight (g)	86.7^{a}	110.9^{b}	136.3^{c}	200.5^{d}	108.7^{b}	131.1^{c}	163.9^{e}	112.3^{b}	133.0^{c}	192.6^{d}	5.86
Gain (g)	-9.3^{a}	14.9^{b}	38.5^{c}	106.4^{d}	10.5^{b}	33.1^{c}	67.8^{e}	15.7^{b}	37.3^{c}	96.4^{d}	5.91
DGC [a] (%/d)	-0.54^{a}	0.81^{b}	1.93^{c}	4.66^{d}	0.57^{b}	1.68^{c}	3.19^{e}	0.85^{b}	1.89^{c}	4.26^{d}	0.33
Survival (%)	100	100	100	100	100	100	100	100	100	100	0.00
Nitrogen retention	0.0^{a}	42.9^{d}	42.6^{d}	37.6^{c}	28.3^{b}	37.6^{c}	31.0^{b}	37.0^{c}	41.5^{cd}	34.6^{bc}	1.01
Energy retention	0.0^{a}	22.4^{c}	42.9^{e}	46.8^{e}	13.2^{b}	31.2^{d}	35.6^{de}	28.2^{d}	43.4^{e}	45.9^{e}	1.75
Feed intake (g/fish)	0.0^{a}	16.2^{b}	32.4^{c}	90.5^{d}	16.2^{b}	32.4^{c}	66.1^{e}	16.2^{b}	32.4^{c}	83.2^{d}	4.71
FCR [b] (g:g)	0.000^{a}	1.093^{c}	0.841^{b}	0.852^{b}	1.562^{d}	0.979^{bc}	0.975^{bc}	1.034^{c}	0.870^{b}	0.862^{b}	0.044
N excreted as Ammonia (%)	68.3^{a}	–	–	79.1^{b}	–	–	70.8^{a}	–	–	77.5^{b}	
N excreted as Urea (%)	31.7^{b}	–	–	20.9^{a}	–	–	29.2^{b}	–	–	22.5^{a}	

[a] Daily Growth Coefficient.

[b] Food Conversion Ratio; grams of dry matter consumed per grams live-weight gain.

[c] Food Conversion Efficiency; grams live-weight gain per dry matter food consumed.

Different superscripts indicated significant ($P < 0.05$) differences among treatments.

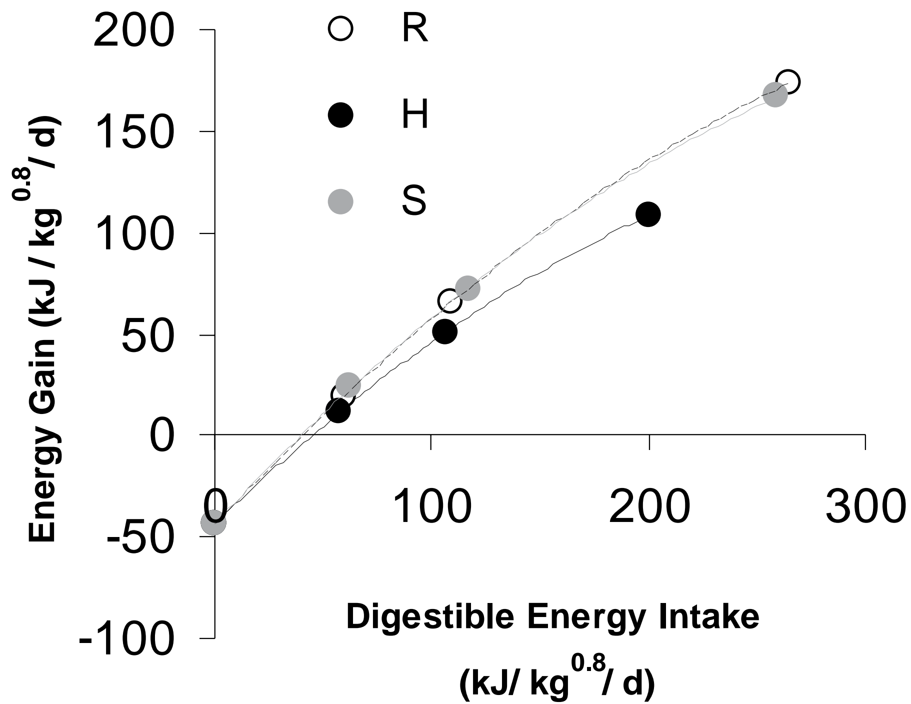

Figure 17.1 Energy retention with varying levels of digestible energy (DE) intake for each treatment (R: Reference, H: heat-dried LPC, S: Spray-dried LPC). Each data point is based on data derived from the mean of four replicates for each diet ration level.

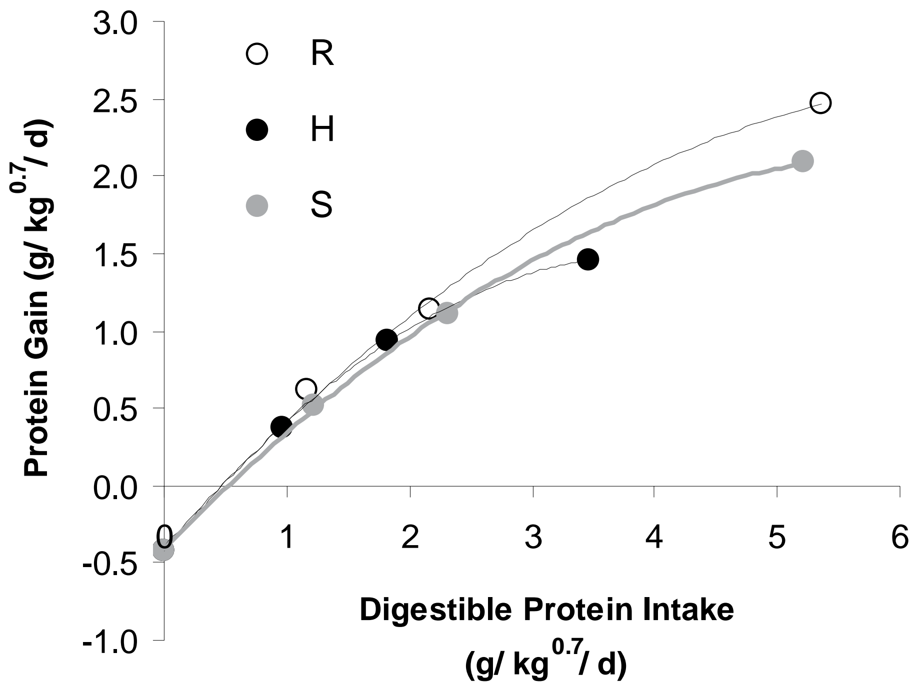

Figure 17.2 Protein retention with varying levels of digestible protein (DP) intake for each treatment (R: Reference, H: heat-dried LPC, S: Spray-dried LPC). Each data point is based on data derived from the mean of four replicates for each diet ration level.

18.0 Developing an in-vitro assessment method for heat damage of proteins and feed quality determination

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Abstract

This chapter describes the development of an alternative technique for the determination of the reactive lysine concentration and nutritional value of prepared aquaculture feed. This work compliments the work reported in other chapters that examines the effect of drying processes on the composition, digestibility and utilisation efficiencies of different aquaculture feeds when fed under controlled conditions.

18.1 Introduction

Amino acids are the basic building blocks of enzymes, proteins, body tissues and some hormones. Amino acids are characterised by the general structure R-CH(NH_2)COOH. That is they comprise an organic chain (R) with an amino group, -NH_2 and a carboxyl group -COOH. Amino acids link to each other when the carboxyl group of one molecule reacts with the amino group of another molecule, creating a peptide bond or amide linkage -C(=O)NH- , releasing a molecule of water in the process. The distinguishing chemistry and function of each amino acid relates to the differing chain denoted by the "R" in the general structure above.

Lysine is an essential amino acid and it is well established that it can be a growth-limiting component in animal diets (Hurrel et al., 1981; Moughan and Rutherfurd, 1996; Williams et al., 2006). The ε-amino group of lysine can interact with carbohydrates in animal feeds to form adducts which are not necessarily available for absorption during digestion.

It is known that during the processing of feeds, lysine can react with other components in the feed material, typically carbohydrates. The adducts thus formed are not easily broken down by digestion rendering lysine as unavailable to the animal (Rutherfurd and Moughan, 1997). This is particularly pronounced where the processing has involved heating, either directly or to a lesser extent by steam or where prolonged storage times of the components have occurred. A typical reaction of this sort is referred to as the Maillard reaction. This is where a reaction occurs between the amino acid and a reducing sugar, usually under the influence of heat. This is enhanced in an alkaline environment because the amino groups are not neutralized. The Maillard reaction is commonly employed to positive effect in the food industry as a result of the flavours that are produced by such reactions (Hurrell et al., 1979; Ames et al., 2005).

Some lysine adducts are reversible under typical protein hydrolysis conditions and this can result in overestimates of the available lysine in feedstuff. This over estimation may result in an unrealistic expectation of animal production from utilisation of such feeds. A more specific analysis is required that does not suffer from such over estimations and can thus be used as a valuable predictor of animal production from feeds.

This chapter describes a component of a larger project, which investigates non-animal sources of protein for use in aquaculture feeds (Glencross et al., 2004; Glencross et al., 2005). The project aims to develop a robust method of assessing feed quality where biologically available

lysine is a principal criterion. This is achieved by measuring the chemically reactive lysine in a variety of feed blends. he fundamental assumption is that lysine within the proteins of the animal feed is available for utilisation if it is also available for chemical reaction. This assumption may be incorrect where the physical access of digestive enzymes is restricted. However, the main factor, which renders lysine nutritionally unavailable, relates to the chemical interaction of the side chain of lysine with matrix components, primarily carbohydrates. In this respect the chemical reactivity of lysine should be a good guide to nutritional availability.

18.2 Methods

The material for analysis is finely ground (< 0.5 mm) and a homogenous sample is treated with alkaline o-methylisourea (OMIU). The reaction is allowed to proceed at room temperature for six days in a sealed container. The resulting sub-sample is then hydrolysed by adding hydrochloric acid and heating at 110°C for 24 hours. The hydrolysed product is then presented to the LC-MS. Blanks, control samples and spiked sample recoveries are determined along with the sample to provide quality assurance.

The method has been optimised to ensure acceptable recoveries (average 85-115%) and to ensure that the relatively small amount of sample in the aquaculture trials was amenable to the procedure. Additionally the procedure has been found to have a dynamic range that allows it to be used in lupin seeds (typically 1-2% lysine), protein concentrates with much higher lysine contents and variable matrix feeds (Glencross et al., 2004). Figure 18.1 shows a typical chromatogram from the procedure.

18.3 Results and Discussion.

There are many analytical chemistry techniques (Moughan and Rutherfurd, 1996; Rutherfurd and Moughan, 1997; Carpenter et al., 1989) available that determine the lysine content in foodstuffs. However, as mentioned above, heat treatment may convert the chemically active lysine to a form that unable to be absorbed in the gut of animals (Ames et al., 2005; Williams et al., 2006). A method that is predictive of the feed trial outcomes will save considerable time and expense as the only real alternative to now has been to conduct costly and time consuming animal trials. Additionally an analytical chemistry based technique will allow feeds and components to be comparatively assessed without the compounding temporal, spatial or animal effects.

The reactive groups in free amino acids include $-NH_2$ and -COOH groups and groups present on side chains. In peptides and proteins only the side chain is available for reactions (besides amino and carboxylic groups at the terminal ends). In lysine-containing proteins, compounds reacting with amino groups can affect both the amino group at N-terminus and the epsilon-amino group of the lysine side chain.

The traditional method of determining chemically reactive lysine is known as the FDNB method. This uses FDNB (1 fluoro- 2, 4, dinitrobenzene), which is also commonly called Sanger's Reagent. The FDNB combines with the ε-amino group of the lysine producing a yellow colour. The intensity of the yellow colour is proportional to the concentration of the reactive lysine.

FDNB-lysine has some significant shortfalls primarily because it is a non-specific, colorimetric assay (Booth, 1971). The products of the Maillard reaction are usually a similar colour, which presents obvious issues. Carrying out additional 'blank' reactions, and then applying correction factors to the result generally overcome these issues. The correction factor is different for

different materials, which makes it difficult to apply to unknown samples or with complex matrices such as feeds. The yellow colour can be overestimated due to pigments produced during hydrolysis or storage of the material and results primarily from carbohydrate related chemistry (Hurrell et al., 1979; Bjarnason and Carpenter, 1970; Boctor and Harper, 1968). In addition, the yellow colour may be underestimated where reducing compounds in the matrix convert the nitro- groups in the FDNB-lysine to amino-groups rendering them colourless. Furthermore, treatment with FDNB can reduce the susceptibility of proteins to hydrolysis. Due to the non-specific nature of the assay and the chemical interferences (particularly those resulting from heat treatment), which can occur, the FDNB assay is not the method of choice for this work. An additional concern is the nature of the FDNB reagent. There is limited evidence to suggest that skin contact of FDNB may produce health effect including hypersensitivity to other irritants and other cumulative health effects. Inhalation or ingestion can cause serious health damage chemical hazards posed by use of FDNB. FDNB is considered to be highly toxic (14). Obviously there is a need to find a technique that avoids the use of this reagent.

The method that has been developed during this research to measure reactive lysine is that primarily based on the work of Moughan and Rutherfurd (1996); with the additional benefit of recent advances in scientific instrumentation, most notably the liquid chromatograph mass spectrometry (LC-MS). The method uses a guanidination reaction utilising o-methyliso urea that has a great specificity for the ε-amino group of lysine. The lysine here is converted to homoarginine. This method has the benefits that include:

- Excellent quantitation,
- Irreversibility under experimental conditions,
- The reaction occurs at room temperature (preventing Maillard reactions that may have occurred during the analytical procedure),
- Amenable to specific (LC-MS) procedures, rather than non specific (e.g. colorimetric) procedures,
- Can be used on relatively small samples (< 0.1g if required),
- Does not use potentially hazardous materials.

The use of LC-MS provides excellent sensitivity, combined with substantially greater confidence in analyte identification compared to chromophore or fluorophore producing derivatisations, with generally equivalent or lower sample handling requirements. The LC-MS is a valuable tool that finds many applications across laboratory activities.

The procedure described below is applicable to material of plant or animal origin. The effect of high fats containing material on the method has not been fully investigated. Samples containing high fat (> 15%) will require solvent extraction prior to analysis. Care must be taken to ensure that lipophilic proteins are not lost during this step.

18.3.1 Conclusions

The relatively small number of analyses carried out to date has indicated that the technique is sufficiently robust to accurately provide a good measure of the reactive lysine in samples of feed and components. The use of the LC-MS has produced excellent results with clear resolution of components and apparent freedom from interferences.

It is acknowledged that a potential interference could come from samples containing homoarginine.

As homoargenine is a toxic amino acid it is not likely that feeds will contain appreciable quantities. Analytical procedures are normally carried out to confirm this as part the routine analysis. Should homoarginine occur in samples a simple correction for this could be applied.

The technique can also be used to quantify the potential protein profile redistribution as a result of the lupin concentrate process (another component of the research group's work). This will determine if it is necessary to supplement the feeds with lysine. If this is found to be necessary is can be done relatively cheaply. Alternatively it may be possible to manipulate the concentrate production technique to avoid losses of lysine during production and processing.

Additional work is yet to be carried to determine the suitability of additional matrices (e.g. faecal material) and the potential losses that may be associated with high fat samples. It is hoped that the data obtained from the current feeding trials will also allow for an assessment of the feed quality by NIR (near infrared spectroscopy). NIR has been used internationally for a number of years for commercially testing the quality of feed such as silage and grains.

The NIR technique is a derivative technique, which allows for calibration against many variables (such as crude protein, dry digestible matter and fibre). It may be possible to use the data from this study to calibrate NIR against the reactive lysine analysis and other biological indicators. If successful the NIR technique is a rapid and relatively simple technique that can be used. The potential down side to the pursuit of the NIR technique may be its robustness. The technique generally requires a relatively constant matrix or at least comprehensive database of matrices for reliable results.

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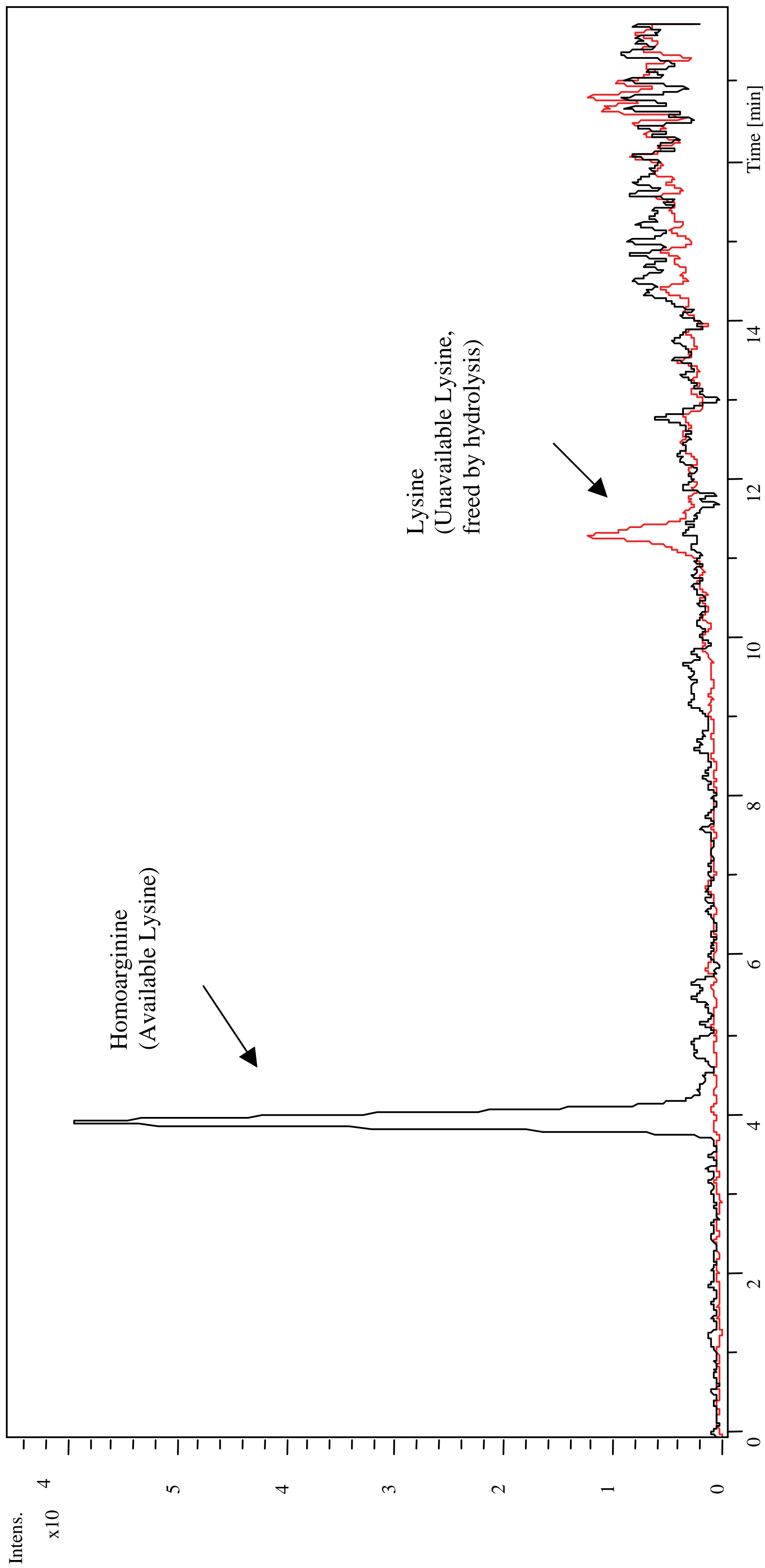

Figure 18.1 Reactive (Available) Lysine in Lupin Protein Isolate.

19.0 A comparison of the digestibility of a range of lupin and soybean protein products when fed to either Atlantic salmon (*Salmo salar*) or rainbow trout (*Oncorhynchus mykiss*)[a]

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Abstract

This study compares the digestibility of a series of lupin and soybean protein products when fed to either rainbow trout or Atlantic salmon. The test ingredients in the study, from one of two key grain resources (lupins: *Lupinus angustifolius* and soybeans), represented various levels of processing of each grain in order to increase the protein content of the meals. A reference ingredient of enzymatically-hydrolyzed casein (EHC) was also included in the study. The rainbow trout (266 ± 18 g) were housed in freshwater tanks (250 l, salinity < 1 ‰, 22.1 ± 1.8°C) and acclimated to the diets for six days before faecal collection commenced. The Atlantic salmon (66 ± 10 g) were housed in similar freshwater tanks (250 l, salinity < 1 ‰, 15°C) and acclimated to the diets for at least six days before faecal collection commenced. Faeces were collected from each fish species using settlement collection methods. The digestibility of organic matter, phosphorus, energy and nitrogen was assessed using the diet-substitution method, with each test ingredient included in the diet at 30%. Several differences were observed between the two fish species in their capacity to digest nutrients and energy from each of the products. Organic matter and energy digestibility of each of the ingredients was largely reflective of the protein content of each ingredient. Protein digestibilities were generally consistent between the two fish species with only lupin kernel meal having a significantly higher digestibility when fed to Atlantic salmon than rainbow trout and the soybean protein concentrate a significantly lower digestibility. Although limited differences in protein digestibility were noted among the ingredients when fed to rainbow trout, more substantial differences were noted when the same ingredients were fed to Atlantic salmon. The digestible energy value of the range of products examined was generally higher in Atlantic salmon than rainbow trout. A clear difference between the two fish species was their capacity to digest phosphorus from the ingredients, with several of the plant protein ingredients showing differences in phosphorus digestibility between the two fish species. Generally, both series of grain products have excellent potential as feed ingredients for either of these species. However, the digestive capacity of Atlantic salmon appears to more positively respond to the absence of dietary non-starch polysaccharide content than that of rainbow trout.

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19.1 Introduction

In an endeavour to reduce the reliance on fish meal in aquaculture diets, numerous nutritional studies have been undertaken on a range of plant protein resources (Moyano et al., 1992; Gomes et al., 1995; Storebakken et al., 1998, 2000). However, modern high nutrient-dense diets for aquatic species have little formulation flexibility to accommodate large amounts of non-useful nutritional content (e.g. fibre or ash). Lupins (*Lupinus* spp.) are reported to have good potential and are gaining popularity as a useful feed ingredient in commercial fish diets (Burel et al., 2000; Farhangi and Carter, 2001; Glencross and Hawkins, 2003). However, similar to most other plant protein resources, lupins also contain a large amount of non-nutritionally useful carbohydrates and even some deleterious carbohydrates that influence the nutritional value of other nutritionally useful components of the ingredient (Francis et al., 2001; Glencross et al., 2003a). Because of these problems, many plant protein resources are not realistic viable alternatives, despite having reasonable protein or energy digestibilities. Furthermore, to address this limitation one option is to process some grain varieties to produce protein concentrate or protein isolate products.

Recent studies have begun to explore the potential of protein-enriched products derived from lupins (Glencross et al., 2003a). Further efforts are now being directed at the development of niche-products such as protein concentrates and isolates which have protein contents ranging from 65 to 90%. How these new products will perform when fed to salmonids has not yet been defined. A range of similar products produced from soybeans exist and have previously been assessed in rainbow trout and Atlantic salmon (Kaushik et al., 1995; Refstie et al., 1998), though not in a combined study. Refstie et al. (2000) did, however, examine the nutritional responses of Atlantic salmon and rainbow trout when fed soybean meal and noted that the two species responded differently to the inclusion of this ingredient. It was suggested that this primary difference was in the capacity of the two species to digest the soybean meal. However, why such a striking difference between two such closely related species exists is unclear and warrants further investigation.

There are several key facets to determining or placing a nutritional or biological value on a feed ingredient, principal of which is defining the proportion of nutrients that an animal can obtain from a particular ingredient through its digestive and absorptive processes. This study reports the digestible value of a variety of protein concentrates and isolates prepared from narrow-leaf lupin and soybean meals when fed to rainbow trout or Atlantic salmon.

19.2 Materials and Methods

19.2.1 Diet development

Digestibility assessment of specific ingredients was undertaken on the diet-substitution basis (Aksnes et al., 1996). A basal diet was formulated and prepared to include a protein level of approximately 480 g/kg DM, a fat level of 175 g/kg DM and an inert marker (chromic oxide at 15 g/kg) (Table 19.1). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added to a sub-sample of the basal mash, (see Table 19.1). The composition of all of the ingredients used is presented in Table 19.2. Each of the test ingredients was thoroughly ground such that all particles passed through an 800 μm sieve. Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw-pressed using a pasta maker through a 3 mm diameter die. The resultant moist pellets were

then oven dried at 90°C for approximately 6 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. Source of all of the ingredients used is presented in Table 19.1. Composition of all diets is presented in Table 19.3.

The lupin protein concentrate and isolate used in this study were prepared in the laboratory using solubilised protein isolation techniques with a basic (pH 9.0) solubilisation of the protein from the lupin kernel meal, followed by filtering of the insoluble components prior to acidification (pH 4.0) of the solution to precipitate the isolate protein. The isolated protein was then neutralised (pH 7.0) prior to be dried using a freeze drier. The concentrate and isolate preparation differed primarily in the stringency used in the filtering process. These techniques were based on those reported by Lasztity et al. (2001).

19.2.2 Fish handling – Rainbow trout

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton strain) (Ward et al., 2003) were transferred from grow-out ponds to experimental tanks (250 L). Freshwater (salinity < 1 ‰) of 22.1 ± 1.8°C at a flow rate of about 4 L/min was supplied to each of the tanks. Each of the tanks were stocked with 6 trout of 266 ± 18 g (mean ± S.D.; n = 32 x 6). Treatments were randomly assigned amongst 32 tanks, with each treatment having four replicates.

Fish were hand fed the diets to apparent satiety once daily at 1800 h. The trout were allowed to acclimatise to the allocated dietary treatment for six days before faecal collection commenced (Wybourne and Carter, 1999). Faeces were collected using settlement techniques based on those reported by Cho and Kaushik (1990). Faeces were collected over five days, pooled within tank, and kept frozen at -20°C before being dried in preparation for analysis.

19.2.3 Fish handling – Atlantic salmon

Hatchery-reared Atlantic salmon (*Salmo salar*) were transferred from SALTAS Pty Ltd Hatchery at Wayatinah, Tasmania, to experimental tanks (250 L) held under constant conditions (Carter and Hauler, 2000). Freshwater (salinity < 1 ‰) of ~15°C at a flow rate of about 3 L/min was supplied to each of the tanks. Each of the tanks were stocked with 36 salmon of 66 ± 10 g (mean ± S.D.; n = 4 x 36). Treatments were randomly assigned amongst 24 tanks, with each treatment having three replicates.

Fish were hand fed the diets to apparent satiety once daily. The salmon were allowed to acclimatise to the allocated dietary treatment for ten days before faecal collection commenced (Wybourne and Carter, 1999). Faeces were collected using settlement techniques based on those reported above. Faeces were collected over six days, pooled within tank, and kept frozen at -20°C before being dried in preparation for analysis.

19.2.4 Chemical and digestibility analysis

Diet and faecal samples were analysed for dry matter, chromium, phosphorus, ash, nitrogen and gross energy content. Samples were freeze-dried prior to analysis. Dry matter content of samples was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Chromium and phosphorus levels were determined following combustion on an ICP mass spectrometer. Protein levels were calculated from the determination of total nitrogen by Leco auto-analyzer, based on %N x 6.25. Fat content of diets was determined gravimetrically following extraction of the lipids according to the method of Folch et al. (1957). Gross ash content was determined

gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Organic matter content was determined based on the difference between dry matter content minus ash content. Gross energy was determined by adiabatic bomb calorimetry. The apparent digestibility coefficient (ADC_{diet}) of each of the key nutritional variables examined was based on the following formula (Maynard and Loosli, 1969):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

Where Cr_{diet} and Cr_{faeces} represent the chromium content of the diet and faeces respectively, and $Nutrient_{diet}$ and $Nutrient_{faeces}$ represent the nutritional parameter of concern (dry matter, protein or energy) content of the diet and faeces respectively (Table 19.5). The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet, respectively (Sugiura et al., 1998).

Ingredient digestibilities that were measured at greater than 100% were not corrected because we believe they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined (Table 19.6). Diet digestibilities are shown in Table 19.5. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0% (Table 19.7).

19.2.5 Statistical analysis

All values are means unless otherwise specified. Data were analysed for homogeneity using Cochran's test. Effects of ingredient on digestibility of organic matter, nitrogen, phosphorus and gross energy in each of the diet were examined by two-way ANOVA with fish species and ingredient set as key factors (Table 19.4). Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

19.3 Results

This study identified several subtle differences in the digestive capacity of the two aquaculture species fed the ingredients used in this study and also in the digestible value of specific ingredients fed to the same fish species. A two-way ANOVA identified effects of fish species, ingredient and an interaction between fish species and ingredient for energy, N, P and organic matter digestibility parameters (Table 19.4). The most notable difference between the two fish species was their differences in digestion of phosphorus from each ingredient. Among the ingredients, substantial improvements in energy and organic matter digestion were noted with increasing level of processing of both lupin kernel meals and soybean meals. However, the influences of processing on phosphorus and nitrogen digestibilities results were not as clear.

19.3.1 Rainbow trout

Within the rainbow trout assessment organic matter digestibilities were significantly lower for ingredients with the lower levels of protein and fat, such as the lupin kernel meal, although digestibility of organic matter was higher from the lupin protein concentrate, soy protein concentrate and soybean meal than the lupin kernel meal. These additional meals were also significantly lower in organic matter digestibility than both protein isolates and the enzymatically-hydrolysed casein (EHC) (Table 19.5). The total levels of digestible organic matter in each of the ingredients increased with increasing protein content. This observation was consistent with both the lupin and soybean ingredients. The highest level of digestible organic matter was observed of the lupin protein isolate (919 g/kg DM), though this was not significantly more than the soybean protein isolate (907 g/kg DM). Both protein isolates had similar digestible organic matter levels to that of the enzymatically-hydrolyzed casein (916 g/kg DM) (Table 19.6).

Phosphorus digestibility was consistently higher in most of the lupin-based ingredients compared to the soybean-based ingredients, lupin protein isolate being the only exception. Phosphorus digestibility of the reference ingredient (EHC) was similar to most of the lupin-based ingredients (Table 19.5). Total levels of digestible phosphorus in each of the lupin products were similar (3.6 g/kg DM to 4.4 g/kg DM), to that of most of the soybean ingredients whose digestible phosphorus content ranged from 3.7 g/kg DM to 5.3 g/kg DM (Table 19.6).

Energy digestibility was significantly increased in both the lupin and the soybean ingredients with increasing protein content of the ingredients. The energy digestibility of the lupin protein isolate was numerically higher than that of the soybean protein isolate, but not significantly so. The energy digestibilities of both the lupin and soybean protein concentrates were similar. The lupin kernel meal and soybean meal energy digestibilities were the lowest of those products assessed, but were not significantly different from each other. Energy digestibility of the EHC was the highest of all ingredients examined (Table 19.5). Total digestible energy was greatest from the soy protein isolate (21.39 MJ/kg DM), though only marginally so from both the lupin protein isolate (21.22 MJ/kg DM) and EHC (20.93 MJ/kg DM) (Table 19.5). Total digestible energy level was lowest in the lupin kernel meal (14.40 MJ/kg DM) which was lower than that of the soybean meal (16.32 MJ/kg DM) (Table 19.6).

There were few significant differences in nitrogen (protein) digestibility among the ingredients examined when fed to rainbow trout. The nitrogen digestibility of the soy protein concentrate (106.9%) was significantly higher than the EHC (96.0%), but there were no other significant differences between ingredients (Table 19.5). Total levels of digestible protein were highest in the soy protein isolate (873 g/kg DM) and lowest in the lupin kernel meal (403 g/kg DM) (Table 19.6).

19.3.2 Atlantic salmon

Within the Atlantic salmon assessment, organic matter digestibilities of the lupin and soybean ingredients meals were generally lower than those observed from the rainbow trout, but not significantly so in any cases (Table 19.5). The organic matter digestibilities of the ingredients, when fed to Atlantic salmon, were highest for the EHC (101.2%) and both the lupin (95.8%) and soybean (97.9%) protein isolates (Table 19.5), but there were no significant differences between ingredients. The overall digestible organic matter in the ingredients was consistent with the digestibilities, in that the EHC and both the protein isolates also had the highest overall digestible organic matter levels and lupin kernel meal the lowest (Table 19.6).

Phosphorus digestibilities were generally significantly poorer for most of the ingredients compared to those phosphorus digestibilities observed of the same products when they were fed to rainbow trout. Within Atlantic salmon, all ingredients were significantly more poorly digested than the EHC (Table 19.5). Total levels of digestible phosphorus in each of the ingredients ranged from 0.0 g/kg DM in the soy protein concentrate to 7.8 g/kg DM in the EHC. Digestible phosphorus levels in each of the lupin based ingredients were highly consistent at around 0.4 to 1.1 g/kg DM (Table 19.6).

Similar to that observed for the rainbow trout, the energy digestibility was significantly improved in both the lupin and the soybean ingredients with increasing protein content of the ingredients. The specific energy digestibilities of the lupin kernel and soybean meals and soy protein concentrate was similar to those observed for rainbow trout, however significant differences were noted between the two species' ability to digest the energy content of each of the higher protein products, with Atlantic salmon showing a better capacity to digest energy from those ingredients (Table 19.5). The total digestible energy levels for Atlantic salmon were marginally different to those observed for rainbow trout, consistent with the slightly different energy digestibilities observed of the suite of ingredients fed to either fish species (Table 19.6).

The nitrogen digestibility of the lupin kernel meal (130.4%) was significantly better that observed for the same ingredient when fed to rainbow trout (Table 19.5). In contrast, the nitrogen digestibility of the soybean protein concentrate was significantly lower when fed to the Atlantic salmon than that observed when it was fed to the rainbow trout. No other significant differences were observed between the two fish species. Within Atlantic salmon, the soy protein concentrate was the most poorly digested ingredient (90.1%), while lupin kernel meal was the best digested (130.4%). No other significant differences within species were noted. Total levels of digestible protein were highest in the EHC and the soy protein isolate (839 and 870 g/kg DM, respectively) and lowest in the lupin kernel meal (415 g/kg DM) (Table 19.6).

19.4 Discussion

This comparison of the nutritional value of a range of lupin and soybean products, when fed to either rainbow trout or Atlantic salmon, highlights the considerable potential of plant meals for use in aquaculture diets for carnivorous fish species. However, the comparison of the two different aquaculture species used in this study shows that despite their taxonomic and physiological similarity, significant differences exist in the capacity of these species to digest certain plant protein resources.

A study by Refstie et al. (2000) compared the utilisation of diets based either on fish meal or soybean meal when fed to Atlantic salmon or rainbow trout. It was noted that the two species responded differently to the dietary inclusion of soybean meal, though differences in the sizes of the animals used in that study, similar to the present one, were also noted. It was suggested that the primary difference was in the capacity of the two species to digest the soybean meal. However, the present study does not necessarily support this finding but does show that some species-specific differences in digestive capacity do exist. The most notable of these differences was the digestibility of phosphorus from each of the products.

It is important to reiterate that in the present study several of the digestibilities were measured at greater than 100%. These have not been corrected because we believe they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined (Table 19.5). However, for reasons of practicality, the total levels of digestible

nutrients and energy (Table 19.6) were only calculated assuming a maximum digestibility of 100% or a minimum of 0%. These vagaries highlight the complex nature of assessing apparent ingredient digestibilities when those ingredients are included in a compounded diet.

19.4.1 Protein concentrates and isolates

The use of plant protein products in aquaculture diets is generally limited by the levels of digestible protein and/or energy in the respective products. Soybeans and lupins represent some of those plant protein products with the higher protein levels and efforts have been made to enhance the protein levels of these grain products through processing. While protein concentrates and isolates are commercially available from soybean derived sources, similar such products produced from lupins are still largely in a development phase. Notable in the products evaluated thus far is an increasing protein content, usually at the expense of the carbohydrate content of the grain. Based on the reports from other studies, it could be reasoned that an increase in the protein content of these grains would be usually concomitant with a decline in the levels of non-starch polysaccharides (NSP) in resultant meals (van Barneveld, 1999; Glencross et al., 2003b).

The development of protein-concentrated plant products shows considerable potential application for the aquaculture sector. Work in this area has been noted since Kaushik et al. (1995) evaluated the digestible value of a wide range of soybean products of various forms when fed to rainbow trout. From that study it was shown that all of the soybean meals had very high protein/nitrogen digestibilities. Clearly those observations are highly consistent with those reported in the present study and the further data on a similar range of lupin-derived products also suggests that this concept may broadly apply across a range of plant protein products.

In the present study a consistent improvement in the digestibility of organic matter and energy was observed with increasing protein content, for both the soybean and lupin products. It is reasoned that this is due to the concomitant decrease in the non-nutritionally useful carbohydrate content of these products. Substantial changes were also observed for phosphorus digestibility in the lupin products when fed to rainbow trout, although in contrast to what was observed for other nutrients, poorer digestibilities were observed with the higher protein, more fully processed lupin products. Phosphorus digestibility of the soybean products was relatively consistent, though generally lower than that of the lupin products. Phosphorus digestibilities of the same products when fed to Atlantic salmon were generally not consistent with protein or processing level.

Other studies with lupin kernel meals have also shown that digestibility of nutrients in this grain is largely influenced by the total carbohydrate content, with decreasing carbohydrate levels being concomitant with increasing protein levels and subsequent improvements in organic matter, energy and protein digestibilities (Glencross et al., 2003b).

19.4.2 Differences in nutritional value between species

The findings of the present study are generally consistent with the observations of Refstie et al. (2000) who also noted differing nutritional responses of Atlantic salmon and rainbow trout when fed soybean meal. These differences are primarily noted in the different capacities that each fish species has in digesting the energy from ingredients with higher protein levels. Interestingly, products with lower protein levels (< 600 g/kg DM) had similar energy digestibilities in both species. This finding provides support for the hypothesis that both species respond similarly to ingredients with high NSP levels, but that Atlantic salmon has a much better capacity to more fully digest the energy content of protein rich ingredients. It could also be argued that dietary NSP restricts the energy digestion of Atlantic salmon more so than that of rainbow trout

as seen by the greater response in energy digestibility in the absence of dietary NSP. Based on the differences seen in energy digestibility it could be suggested that rainbow trout are a more sensitive species in assessing ingredient digestibility quality between the two fish species, based on the observation that they did not respond to the reduction in NSP/increase in protein as well as the Atlantic salmon did.

Comparisons of earlier digestibility estimates between the two fish species, based on comparisons of Atlantic salmon data by Refstie et al. (1998) also differ markedly from the rainbow trout data of Kaushik et al. (1995) who evaluated some similar meals. However, such a comparison highlights the many problems of inter-study comparisons, as variability in fish management practices, analytical methods, specific ingredient composition and faecal collection methods effectively confounds the validity of such comparisons (Vandenberg and de la Noue, 2001). In the present study considerable effort has been made to ensure that not only the same methods and ingredients were used, but also the same diets. Furthermore, a reference ingredient (EHC) available from an international pharmaceutical supplier was used and is suggested as a standard reference for such studies to allow for subsequent inter-study comparisons.

Considerable differences were also noted between the two fish species in their capacities to digest phosphorus from each of the ingredients. For most ingredients, the digestibility of phosphorus by rainbow trout was considerably more than that observed for Atlantic salmon. The specific reasons for this are unclear and need further investigation.

19.4.3 Conclusions

The range of plant protein products evaluated in this study shows clear potential for both rainbow trout and Atlantic salmon. Subtle differences in the digestive capacities of each fish species are apparent and the results suggest that Atlantic salmon have a slightly better capacity to deal with higher protein content ingredients than rainbow trout. Despite the subtle differences in the digestibilities of the plant protein products between the two fish species, both fish species can derive good nutritional value from the ingredients studied, but in formulating diets care needs to be taken to use the species specific data to ensure that diets are equivalent on a digestible nutrient basis.

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Table 19.1 Formulations of the experimental diets (all values are g/kg).

Ingredient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
Fish meal [a]	650	455	455	455	455	455	455	455
Fish oil [a]	110	77	77	77	77	77	77	77
Lupin kernel meal [b]	0	300	0	0	0	0	0	0
Lupin protein concentrate [c]	0	0	300	0	0	0	0	0
Lupin protein isolate [c]	0	0	0	300	0	0	0	0
Soybean [a]	0	0	0	0	300	0	0	0
Soy protein concentrate [d]	0	0	0	0	0	300	0	0
Soy Protein Isolate [e]	0	0	0	0	0	0	300	0
Enzymatically hydrolized casein (EHC)[e]	0	0	0	0	0	0	0	300
Pregelled wheat starch [f]	150	105	105	105	105	105	105	105
Cellulose [e]	65	46	46	46	46	46	46	46
Vitamin and mineral premix [g]*	10	7	7	7	7	7	7	7
Chromic oxide [h]	15	11	11	11	11	11	11	11

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia.

[b] Supplied by WESFEEDS Pty Ltd, Welshpool, Western Australia, Australia.

[c] Supplied by Department of Agriculture, South Perth, Western Australia, Australia.

[d] (HP300) Supplied by Hamlet-Protein AS, Horsens, Denmark.

[e] Supplied by ICN Biomedical, Costa Mesa, CA, USA.

[f] Supplied by Weston BioProducts, Henderson, Western Australia, Australia.

[g] Supplied by Rhone Poulenc, Goodna, Queensland, Australia. [h] Supplied by SIGMA, St Louis, Missouri, United States.

* Vitamin and mineral premix includes (IU kg^{-1} or g/kg of premix): Vitamin A, 2.5 MIU; Vitamin D^3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K^3, 1.7 g; Thiamine (Vitamin B1), 2.5 g; Riboflavin (Vitamin B2), 4.2 g; Niacin (Vitamin B3), 25 g; Calcium Pantothenate (Vitamin B5), 8.3; Pyridoxine HCl (Vitamin B6), 2.0 g; Folate (Vitamin B9), 0.8; Cyanocobalamin (Vitamin B12), 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g).

Table 19.2 Nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated).

Nutrient	[a]Lupin kernel meal	[a]Lupin protein concentrate	[a]Lupin protein isolate	[b]Soybean meal	[c]Soy protein concentrate	Soy protein isolate	[d]EHC	Fish meal	Pregelatinsed wheat starch	Cellulose
Dry matter content (g/kg)	885	942	926	909	939	938	916	917	906	942
Crude protein	415	690	810	518	590	893	839	770	7	7
Crude fat	53	93	125	47	54	13	11	68	11	11
Ash	33	31	30	69	79	47	70	142	3	2
Phosphorus	4	5	5	8	9	9	9	22	1	0
Gross energy (MJ/kg DM)	20.42	22.23	22.62	19.58	20.29	22.98	21.19	21.28	17.19	17.00

[a] All lupin products are derived from *Lupinus angustifolius* cv. Gungarru.

[b] Soybean meal is heat-treated, solvent-extracted, dehulled soybean meal.

[c] Soy protein concentrate is HP300.

[d] Enzymatically Hydrolysed Casein.

Table 19.3 Nutrient composition of the experimental diets (all values are g/kg DM, unless otherwise indicated).

Nutrient	Diet 1	Diet 2	Diet 3	Diet 4	Diet 5	Diet 6	Diet 7	Diet 8
Dry matter content (g/kg)	963	962	961	961	959	947	962	951
Crude protein	476	457	546	577	492	521	600	580
Crude fat	174	146	110	132	129	128	121	126
Ash	108	87	114	85	100	101	91	97
Phosphorus	16	14	12	12	13	13	13	13
Gross energy (MJ/kg DM)	22.64	22.13	22.41	22.60	21.86	21.88	22.52	22.19

Table 19.4 Two-way ANOVA table for statistical parameters of fish species and ingredient, with additional one-way ANOVA tables for ingredient differences within fish species.

	Parameter	SS	DoF	MS	F	p
Fish species	Organic matter	0.001	1	0.001	0.96	0.333
Ingredient	Organic matter	0.818	6	0.136	112.68	0.000
Fish species x Ingredient	Organic matter	0.026	6	0.004	3.53	0.008
Fish species	Phosphorus	2.435	1	2.435	100.02	0.000
Ingredient	Phosphorus	1.710	6	0.285	11.70	0.000
Fish species x Ingredient	Phosphorus	0.779	6	0.130	5.33	0.001
Fish species	Energy	0.054	1	0.054	44.37	0.000
Ingredient	Energy	0.411	6	0.068	55.95	0.000
Fish species x Ingredient	Energy	0.031	6	0.005	4.17	0.003
Fish species	Nitrogen	0.002	1	0.002	3.43	0.073
Ingredient	Nitrogen	0.010	6	0.002	3.03	0.017
Fish species x Ingredient	Nitrogen	0.022	6	0.004	6.47	0.000
		Value	**F**	**Effect**	**Error**	**p**
Atlantic salmon						
Ingredient	Organic matter	0.465	6	0.077	101.42	0.000
	Phosphorus	1.455	6	0.242	7.45	0.001
	Energy	0.238	6	0.039	58.06	0.000
	Nitrogen	0.024	6	0.004	3.83	0.018
Rainbow trout						
Ingredient	Organic matter	0.365	6	0.061	40.32	0.000
	Phosphorus	0.963	6	0.161	8.50	0.000
	Energy	0.197	6	0.033	20.79	0.000
	Nitrogen	0.005	6	0.001	3.60	0.012

Table 19.5 Digestibility (%) of the experiment diets.

Nutrient	Reference	Lupin kernel meal	Lupin protein concentrate	Lupin protein isolate	Soybean meal	Soy protein concentrate	Soy protein isolate	EHC	Pooled SEM
Rainbow trout									
Organic Matter	85.8	79.3	85.7	88.8	83.6	84.9	88.9	89.8	0.61
Phosphorus	47.2	60.2	54.5	51.7	51.2	51.4	48.6	57.6	0.90
Energy	87.6	82.2	87.7	89.6	85.9	87.2	90.1	90.8	0.50
Nitrogen	93.9	95.0	95.7	95.8	94.9	96.4	95.9	95.7	0.16
Atlantic salmon									
Organic Matter	82.6	74.3	82.7	87.1	81.9	81.3	87.1	88.8	0.90
Phosphorus	58.6	53.6	50.3	52.7	50.4	43.0	52.2	63.9	1.48
Energy	85.0	79.1	86.0	89.2	83.7	85.1	89.4	90.6	0.75
Nitrogen	91.8	91.6	93.1	94.2	90.5	91.2	93.9	94.9	0.35

Table 19.6 Digestibility (%) of the test ingredients.

Nutrient	Lupin kernel meal	Lupin protein concentrate	Lupin protein isolate	Soybean meal	Soy protein concentrate	Soy protein isolate	EHC	Pooled SEM
Rainbow trout								
Organic Matter	$^{a}64.8^{c}$	$^{a}76.7^{b}$	$^{a}94.8^{a}$	$^{a}77.3^{b}$	$^{a}82.0^{b}$	$^{a}95.2^{a}$	$^{a}98.5^{a}$	1.84
Phosphorus	$^{a}272.2^{a}$	$^{a}87.2^{a}$	$^{a}71.7^{b}$	$^{a}56.7^{b}$	$^{a}58.9^{b}$	$^{a}42.2^{b}$	$^{a}85.4^{a}$	11.77
Energy	$^{a}70.5^{d}$	$^{b}86.6^{b}$	$^{b}93.8^{ab}$	$^{a}83.3^{cd}$	$^{b}85.6^{bc}$	$^{b}93.1^{ab}$	$^{b}98.8^{a}$	1.42
Nitrogen	$^{b}97.2^{ab}$	$^{a}101.0^{ab}$	$^{a}98.6^{ab}$	$^{a}99.0^{ab}$	$^{a}106.9^{a}$	$^{a}97.8^{ab}$	$^{b}96.0^{b}$	0.57
Atlantic salmon								
Organic Matter	$^{a}55.3^{d}$	$^{a}81.8^{b}$	$^{a}95.8^{a}$	$^{a}73.4^{c}$	$^{a}78.3^{cb}$	$^{a}97.9^{a}$	$^{a}101.2^{a}$	3.42
Phosphorus	$^{b}26.0^{b}$	$^{b}8.1^{b}$	$^{a}22.6^{b}$	$^{a}8.1^{b}$	$^{b}-20.4^{b}$	$^{a}24.2^{b}$	$^{a}91.4^{a}$	8.05
Energy	$^{a}69.6^{c}$	$^{a}105.9^{a}$	$^{a}104.5^{a}$	$^{a}89.0^{b}$	$^{a}101.2^{a}$	$^{a}117.4^{a}$	$^{a}109.3^{a}$	3.32
Nitrogen	$^{a}130.4^{a}$	$^{a}108.7^{a}$	$^{a}96.9^{ab}$	$^{a}94.4^{ab}$	$^{b}90.1^{b}$	$^{a}97.4^{ab}$	$^{a}112.4^{a}$	2.95

Different pre superscripts within columns indicate significant differences between means among fish species but within nutrients and ingredients ($P < 0.05$).
Different post superscripts within rows indicate significant differences between means among ingredients, but not between nutrients or fish species ($P < 0.05$).
EHC: Enzymatically-hydrolysed casein. Pooled SEM: Pooled Standard Error of the Mean.

Table 19.7 Digestible nutrient content (g/kg DM, unless otherwise detailed) of the test ingredients. Data derived from Tables 19.2 and 19.6.

Nutrient	Lupin kernel meal	Lupin protein concentrate	Lupin protein isolate	Soybean meal	Soy protein concentrate	Soy protein isolate	EHC
Rainbow trout							
Organic Matter	627	743	919	720	756	907	916
Phosphorus	3.7	4.4	3.6	4.4	5.3	3.7	7.3
Energy	14.40	19.24	21.22	16.32	17.36	21.39	20.93
Protein	403	690	799	513	590	873	806
Atlantic salmon							
Organic Matter	535	792	929	683	721	933	930
Phosphorus	1.0	0.4	1.1	0.6	0.0	2.1	7.8
Energy	14.21	22.23	22.62	17.43	20.29	22.98	21.19
Protein	415	690	784	490	531	870	839

20.0 A comparison of the digestibility of grain protein products, when fed to rainbow trout (*Oncorhynchus mykiss*) or Atlantic salmon (*Salmo salar*) at different temperatures, in extruded diets

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Abstract

This study compared the diet and ingredient digestibilities of diets fed to either Atlantic salmon or rainbow trout from three independent studies. Each study used either Atlantic salmon or rainbow trout at a warm water (~15°C) or in cold water (~6°C) to examine the digestibility of a common set of diets made using extrusion technology and including a 30% component of a test ingredient. Diet digestibilities for both nitrogen and energy were higher for the rainbow trout at 15°C than the Atlantic salmon at 6°C. The higher diet digestibilities were also consistent with higher ingredient nitrogen and energy digestibilities. Digestibility of the nitrogen content of the diets was lower in the Atlantic salmon at 15°C than those observed of the Atlantic salmon at 6°C. Ingredient digestibilities were also lower for the Atlantic salmon at 15°C. Correlations between diet nitrogen digestibilities were strong among all three experiments and for diet energy digestibilities between the Atlantic salmon at 6°C and rainbow trout. Correlations between ingredient nitrogen digestibilities were strong between the Atlantic salmon at 6°C and rainbow trout, but not between the Atlantic salmon at 15°C and either of the other two experiments. Correlations between ingredient energy digestibilities were also strong between the Atlantic salmon at 6°C and rainbow trout. The findings of this study show that there are strong correlations between species, but not necessarily within species. Water temperature is also shown to be potentially influential. The lack a correlation between ingredient digestibilities within the same species, but at different temperatures supports the need for such trials to be wholly conducted within the one laboratory to minimise inter-laboratory variance.

20.1 Introduction

There is a considerable volume of work on the nutritional value to salmonids of grain products produced from soybean, peas and lupins in both extruded and un-extruded diets (Kaushik et al., 1995; Refstie et al., 1998; Burel et al., 2000; Carter and Hauler, 2000; Glencross and Hawkins, 2004; Glencross et al., 2004a; 2004b). Most of these works have shown that there are clear advantages to extruding some raw materials, with improvements in dry matter and energy digestibilities, but notably the ingredients that are improved tend to be ones with a high starch content and/or significant levels of heat-labile anti-nutritional factors (ANF). Lupins by contrast have essentially no starch content or heat-labile ANF (Petterson, 2000).

Even though rainbow trout, *Oncorhynchus mykiss* and Atlantic salmon, *Salmo salar*, are both from the same family of fish (*Salmonidae*), there have been mixed reports about the homology in nutritional responses by the two species when fed similar raw materials (Refstie et al., 2000;

Glencross et al., 2004a). Studies by Refstie et al. (2000) compared the nutritional responses of Atlantic salmon and rainbow trout when fed soybean meal and noted that the two species responded differently to the inclusion of this ingredient. It was suggested that this primary difference was in the capacity of the two species to digest the soybean meal. Glencross et al. (2004a) questioned the basis for differences in digestibility of raw materials between the two fish species based on their similar phylogenetic and physiological backgrounds. To examine this issue the digestibility of lupin and soybean meals, concentrates and isolates was compared from the same diets using the same faecal collection methods and found a high degree of correlation in responses to energy digestibilities by each species. However, no significant correlation was observed for nitrogen digestibilities between the two species (Glencross et al., 2004a). It was suggested that the nitrogen digestibility correlations were subject to greater error through limited variability and that this had reduced the correlation observed between the two species.

Krogdahl et al. (2004) compared the digestion, utilisation on several metabolic factors of both Atlantic salmon and rainbow trout fed high and low corn starch diets. It was shown that the growth responses of each species were quite similar as were the energy and protein retention features. Rainbow trout digestibilities were slightly higher than those of Atlantic salmon and the Atlantic salmon digestibilities were more influenced by the inclusion of starch. However, differences in diet digestibilities were also noted between the freshwater and seawater maintained fish in this work (Krogdahl et al., 2004).

This study examines a comparison in the digestible value of diets and their component test ingredients when fed to rainbow trout or Atlantic salmon. The data is derived from three separate studies undertaken by three independent laboratories, each evaluating the same diets but with either different species or under different water temperatures or salinities (Refstie et al., 2006; Chapter 5, Chapter 21). The specific features of the raw materials being evaluated are not discussed in this chapter as they have been detailed elsewhere. This comparison was done to examine the transferability of data for one species to the other and the robustness of inter-laboratory comparisons of digestibility assessments.

20.2 Materials and Methods

20.2.1 Ingredient and diet development

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For this, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 20.1). A 1500 kg batch of a basal mash was prepared from a single batch of ingredients and thoroughly mixed and milled through a 750 µm hammermill, forming the basis for all experimental diets in this study. The ingredient of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 20.1). The composition of each test and basal mash ingredient is presented in Table 20.2. The basal diet was prepared without the addition of any test ingredient.

Diets were processed by extrusion through a laboratory scale Wenger X185 extruder at the Australasian Experimental Stockfeed Extrusion Centre (AESEC). Diets made using extrusion were initially preconditioned with the addition of steam, prior to entry of the mash to the barrel. Barrel temperatures were set at 80, 100 and 140°C from entry to die respectively. Water was also injected into the barrel. A standard salmonid feed screw configuration was used (Evans,

1998). After exit from the die (5mm) the extrudate was cut to produce pellets. The pellets were then dried on a counter-flow heated air drier. Diets were made without the oil component added to the mash. The allotted oil component of each diet was vacuum infused to the pellets following pellet drying.

20.2.2 Fish handling

Batches of the experimental feeds were sent to three different laboratories for assessment in Atlantic salmon and rainbow trout. Rainbow trout digestibility analysis was undertaken by the Department of Fisheries at their Pemberton Freshwater Research Centre, in Western Australia. Atlantic salmon digestibility analysis was undertaken at 6°C by AKVAFORSK at their Sunndalsøra laboratory in Norway. The School of Aquaculture – University of Tasmania, at their Launceston laboratory in Tasmania, Australia, undertook Atlantic salmon digestibility analysis at 15°C.

20.2.2.1 Rainbow trout handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 l). Freshwater (salinity < 1 PSU; Dissolved oxygen 7.0 ± 0.5 mg/L) of 16.0 ± 0.1°C (mean ± S.D.) at a flow rate of about 4 l/min was supplied to each of the tanks. Each of the tanks were stocked with 15 trout of 263.4 ± 45.8 g (mean ± S.D.; n = 40). Treatments were randomly assigned amongst 24 tanks, with each treatment having three replicates. Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600 each day. The trout were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and stored in a freezer at -20°C. Stripped faeces were collected during 0800 to 1000 over a four-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples from different days were pooled within tank, and kept frozen at -20°C before being freeze-dried in preparation for analysis.

20.2.2.2 Atlantic salmon handling and faecal collection – Cold water

The experiment was conducted in accordance with laws and regulations that control experiments and procedures in live animals in Norway, as overseen by the Norwegian Animal Research Authority. The experiment was done at AKVAFORSK (Sunndalsøra, Norway), where seawater adapted Atlantic salmon (*Salmo salar*) were fed the experimental diets for a total of 22 days. Prior to the experiment the fish were fed commercial diets (Skretting AS, Stavanger, Norway). At the onset of the experiment, 21 groups of salmon (176 g, 118 fish/group) were randomly distributed from a holding tank to fibreglass tanks (1 x 1 x 0.6 m, water depth 40-50 cm) supplied with seawater, and the experimental diets were randomly allocated to three groups of fish each. The fish were then fed the experimental diets for 21 feeding days. The fish were fed continuously (24 hr d^{-1}) by electrically driven disc feeders, aiming for 15% overfeeding based on expected feed intake. The water temperature during the experimental period was stabilised at 5.6°C, and the O_2 saturation of the outlet water was above 80%. Faeces were stripped from fish in each tank as described by Austreng (1978). The faecal samples were pooled per tank and immediately frozen and stored at -20°C.

20.2.2.3 Atlantic salmon handling and faecal collection – Hot water

All female pre-smolt Atlantic salmon were obtained from the Huon Aquaculture Company (Tasmania, Australia) over 3 weeks (farm weight estimate, 493 ± 42 g). Fish were held at the School of Aquaculture in six 2000-L Rathburn tanks that were each a self-contained partial recirculation system equipped with physical, biological and UV filtration. Water temperature was controlled at 15.0 ± 1.5°C and fish were exposed to ambient photoperiod. Water quality was maintained within recommended limits (Tarazona and Munoz 1995). A commercial salmon feed was hand fed 2-3 times per day for over an acclimation period of 4 to 6 weeks. At the start of the apparent digestibility experiment all diets were hand fed three times a day to appetite and feed intake estimated from the weight of pellets fed. The six diets were randomly allocated to one group in each of three time periods. Diets were fed for 7 days and the salmon stripped (Austreng 1978; Percival et al. 2001) on day 8 in the morning. In order to randomise the effects of previous diets the fish were re-mixed during re-allocation to tanks. Groups were fed the commercial feed for 6 to 7 days and then transferred to the experimental diet for a further 7 days. Following initial sampling salmon were reused twice to obtain triplicate samples for each diet. Faecal samples were freeze-dried pooled into one sample prior to analysis.

20.2.3 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium, ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Total lipid content of the diets was determined gravimetrically following extraction of the lipids according to the Folch method. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

Where Y_{diet} and Y_{faeces} represent the chromium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included

in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined. However, for reasons of practicality, the total levels of digestible nutrients/energy were only calculated assuming a maximum digestibility of 100% or a minimum of 0%.

20.2.4 Statistical analysis

All values are means unless otherwise specified. Correlation analysis was performed using Statistica v6. Curve fitting of linear regressed relationships was undertaken using both Microsoft Excel and Statistica v6. Levels of significance were determined using a Least Significant Difference (LSD) test. Limits for all critical ranges were set at $P < 0.05$.

20.3 Results

20.3.1 Diet digestibilities

There were several differences among the diet digestibility parameters between the three experiments (Table 20.3). Diet nitrogen digestibilities (mean ± SD) were highest in the experiment with the rainbow trout (0.909 ± 0.012) and lowest in the Atlantic salmon at 15°C (0.805 ± 0.033). Diet energy digestibilities were highest in the experiment with the rainbow trout (0.879 ± 0.035) and lowest in the Atlantic salmon at 6°C (0.788 ± 0.036), although no data was available for the Atlantic salmon at 15°C.

The significance of the correlations between the digestibilities of the diets varied between each of the different assessments (Table 20.3; Table 20.4). The strongest correlations were those between the rainbow trout and Atlantic salmon at 6°C for nitrogen (R^2=0.978) (Table 20.4, Figure 20.1). The correlation between diet nitrogen digestibilities for Atlantic salmon at 6°C and the Atlantic salmon at 15°C was lower, but still strong (R^2=0.883). Diet nitrogen digestibilities were generally better correlated than the diet energy digestibilities, but with only a single data-set for diet energy digestibilities, comparison between these parameters lacks any power.

20.3.2 Ingredient digestibilities

There was substantial variation in the digestibility parameters between the three experiments (Table 20.3). Ingredient nitrogen digestibilities (mean ± SD) were highest in the experiment with the rainbow trout (0.947 ± 0.033) and lowest in the Atlantic salmon at 15°C (0.817 ± 0.079). Ingredient energy digestibilities were highest in the experiment with the rainbow trout (0.822 ± 0.080) and lowest in the Atlantic salmon at 6°C (0.782 ± 0.120), although no data was available for the Atlantic salmon at 15°C.

There was limited correlation between the digestibilities of the ingredients between the three experiments (Table 20.3). The strongest correlations were those between the rainbow trout and Atlantic salmon at 6°C for energy (R^2=0.850) (Table 20.4, Figure 20.1). Diet nitrogen

digestibilities were poorer correlated than the diet nitrogen digestibilities, but with only a single data-set for diet energy digestibilities, comparison between these parameters lacks any power.

20.4 Discussion

This study examined a comparison in the digestible value of diets and their component test ingredients when fed to rainbow trout, *Oncorhynchus mykiss* or Atlantic salmon. The data was derived from three separate studies undertaken by three independent laboratories, each evaluating the same diets but with either different species or under different water temperatures (Refstie et al., 2006; Chapter 5, Chapter 21). This comparison builds on from earlier work that examined the digestibility of a series of lupin and soybean products when fed to rainbow trout and Atlantic salmon by the same group of researchers (Glencross et al., 2004a). Notably, the size of fish used in the present study is more uniform (263g, 176g, 493g cf. 66g, 266g) than that reported in the study by Glencross et al. (2004a). However, other factors such as water salinity and temperature were introduced as a by-product of the role of these works in other experiments (Refstie et al., 2006; Chapter 5, Chapter 21). However, despite these differences a comparison of the three experiments provides some insight into the scope and limitations of cross-experimental comparisons.

20.4.1 Diet digestibility effects

The study by Refstie et al. (2000) compared the utilisation of diets based either on fish meal or soybean meal when fed to Atlantic salmon or rainbow trout. It that study the two species responded differently to the dietary inclusion of soybean meal, though differences in the sizes of the animals used in that study, were also noted. It was suggested that the primary difference was in the capacity of the two species to digest the soybean meal. However, the present study does not necessarily support this finding but does show that some species-specific differences in digestive capacity do exist. This hypothesis was also countered by the findings of Glencross et al. (2004a) who showed a strong correlation between the digestibilities of Atlantic salmon and rainbow trout fed the same diets. In a later study it was suggested by Krogdahl et al. (2004) that Atlantic salmon and rainbow trout metabolised nutrients differently. In that study similar protein and energy digestibilities were also observed between the two species for most nutrients, but not for starch. Significant effects of freshwater and saltwater were also noted, which is relevant to the present study as the Atlantic salmon in both cases were in saltwater while the rainbow trout were in freshwater.

Correlation between the experiments was better for the diet digestibilities than those for the ingredients. This is to be expected given the potential for compounding of errors associated with the process for determining ingredient digestibilities. The diet nitrogen digestibilities correlated better than those of the diet energy digestibilities, but with only a single correlation value for the energy digestibilities such a comparison lacks a lot of power.

Of interest was the observation that the poorest diet digestibilities were those from the Atlantic salmon at 15°C and that these were lower than those from fish fed the same diets at 6°C (Table 20.3). This is contrary to the findings of other studies where an increase in temperature resulted in an increase in digestibilities. Windell et al. (1978) noted an influence of water temperature (7°C and 15°C) on dry matter, protein, lipid, carbohydrate or energy digestibility of diets fed to rainbow trout. In addition, substantial differences were noted in the digestibility of starch of varying levels of gelatinization between rainbow trout (*Oncorhynchys mykiss*) held at either 8°C or 18°C (Kaushik, 2001). The digestibility values from the Atlantic salmon at 15°C in this

study are also substantially lower than those reported by Glencross et al. (2004a) for Atlantic salmon at a similar temperature fed similar ingredients, but notably a different faecal collection methods was used in each case and this has been shown to have significant effects on the digestibility determinations of both diets and ingredients (Glencross et al., 2005).

20.4.2 Ingredient digestibility effects

Correlations between experiments for ingredient digestibilities were always weaker than those for the corresponding diet digestibilities. Of the ingredient digestibilities the only significant ingredient digestibility correlations were those between the Atlantic salmon at 6°C and the rainbow trout for both ingredient nitrogen and energy digestibilities. This is highly consistent with earlier reports by Glencross et al. (2004a) that showed an eve n stronger correlation between the two species for a range of lupin and soybean based ingredients (Figure 20.4). The lack of a significant correlation between the two Atlantic salmon studies is unusual as it was expected to be more strongly correlated than those between the Atlantic salmon and the rainbow trout. There was also poor correlation between the Atlantic salmon at 15°C and the rainbow trout for ingredient digestibilities.

The corresponding ingredient digestibility correlations within an experiment comparison were stronger (albeit n=1) for energy digestibilities. This is probably due to the greater differences observed in the ingredient energy digestibilities for both species, than those observed for nitrogen digestibilities allowing greater power to be used in the regression analysis.

Additional comparisons of earlier digestibility estimates between the two fish species, based on comparisons of Atlantic salmon data by Refstie et al. (1998) also differ markedly from the rainbow trout data of Kaushik et al. (1995) who evaluated some similar meals. However, such a comparison highlights the many problems of inter-study comparisons, as variability in fish management practices, analytical methods, specific ingredient composition and faecal collection methods effectively confounds the validity of such comparisons (Vandenberg and de la Noue, 2001; Glencross et al., 2005). While in the present study effort has been made to ensure that similar methods, diets and ingredients were used, the outcomes still highlight the difficulties in obtaining robust assessment through such an inter-laboratory evaluations.

20.4.3 Conclusions

The findings of this study show that there are considerable differences between different laboratories assessing the same feeds, even in the same fish species, albeit at different temperatures. This finding supports that the most robust comparisons are likely to be ones made within the same laboratory as demonstrated by the comparison of the findings from the present study compared with those of Glencross et al. (2004a) and Krogdahl et al., (2004). Although the differences among these inter-laboratory studies make it difficult to confirm digestibility differences or similarities of different grain products by the two species (Atlantic salmon and rainbow trout), the observed level of correlation in this and other studies does suggest that there are similarities between the two species and the diet digestibilities and in some cases ingredient digestibilities from one species may be applicable to the other, at least in relative terms. However, a further work specifically examining this issue would be well to consider a more complete factorial approach to water temperature, salinity and fish species being conducted by a single laboratory.

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Table 20.1 Formulations and composition of the experiment diets (all values are g/kg).

	Reference Diet	SOY	APC	LPC	LKM	BKM	MKM
Ingredient							
Fishmeal	700.0	490.0	490.0	490.0	490.0	490.0	490.0
Fish oil	150.0	105.0	105.0	105.0	105.0	105.0	105.0
Soybean meal		300.0					
Angustifolius Protein Concentrate			300.0				
Luteus Protein Concentrate				300.0			
L. luteus cv Wodjil kernel meal					300.0		
L. angustifolius cv. Belara kernel meal						300.0	
L. angustifolius cv. Myallie kernel meal							300.0
Wheat flour	144.0	100.8	100.8	100.8	100.8	100.8	100.8
Vitamin and mineral premix	5.0	3.5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1.0	0.7	0.7	0.7	0.7	0.7	0.7
Diet composition as analysed							
Dry matter	935	938	938	944	925	924	929
Protein	514	512	584	593	507	508	478
Fat	216	156	190	184	186	159	177
Phosphorus	18	15	15	15	15	14	15
Ash	124	108	92	93	98	99	96
Gross Energy	23.5	22.4	23.7	23.6	23.1	22.1	22.8

[a] From *L. luteus* (yellow lupins).

[b] From *L. angustifolius* (Sweet lupins).

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Table 20.2 Nutrient composition of the experimental ingredients (all values are g/kg DM unless otherwise indicated).

Nutrient	[a] Fish meal	[a] Wheat	Soybean [b]	APC [c]	LPC [d]	LKM [e]	BKM [f]	MKM [e]
Dry matter content (g/kg)	931	905	907	926	932	909	918	905
Crude protein	749	142	521	783	811	537	452	425
Crude fat	87	24	19	110	55	77	80	75
Ash	161	11	69	29	32	44	34	34
Phosphorus	28	2	8	7	8	7	5	5
Gross energy (MJ/kg DM)	20.5	18.4	19.3	25.1	24.1	21.1	20.2	20.8
Arginine	41	7	3.37	7.59	7.33	5.35	46	4.17
Histidine	13	1	1.30	1.67	1.76	1.43	8	1.08
Isoleucine	29	5	2.26	3.30	2.90	2.06	17	1.66
Leucine	56	10	4.06	5.82	6.51	4.41	31	2.91
Lysine	55	5	2.88	3.32	3.10	2.71	22	1.68
Methionine	21	2	0.82	0.56	0.62	0.47	3	0.33
Phenylalanine	30	6	2.72	3.33	3.22	2.24	17	1.76
Threonine	32	5	2.14	2.54	2.34	1.92	16	1.51
Valine	33	6	2.13	2.57	2.28	1.70	17	1.40

[a] Fish meal: Chilean anchovy meal and Australian feed grade wheat, Skretting Australia, Cambridge, TAS, Australia.

[b] Solvent extracted soybean meal (US origin), WESFEEDS, Bentley, WA, Australia.

[c] APC: *L. angustifolius* Protein Concentrate, Department of Agriculture, South Perth, WA, Australia.

[d] LPC: *L. luteus* Protein Concentrate, Department of Agriculture, South Perth, WA, Australia. [f] LKM: Yellow lupin: *L. luteus* Kernel Meal and MKM Sweet lupin: *L. angustifolius* cv Myallie Kernel Meal, Coorow Seed Cleaners, Coorow, WA, Australia.

[e] BKM: Sweet lupin: *L. angustifolius* cv Belara Kernel Meal, Department of Agriculture, South Perth, WA, Australia.

Table 20.3 Digestibility (%) specifications of diets and test ingredients as determined from diets that were processed using either extrusion or screw-press technologies.

	Reference	Soybean	APC	LPC	AKM	BKM	LKM	Pooled SEM
Diet Digestibility – Rainbow trout (16°C)								
Energy	0.910	0.850	0.913	0.906	0.831	–	0.862	0.013
Protein	0.899	0.901	0.925	0.921	0.899	–	0.906	0.004
Ingredient Digestibility – Rainbow trout (16°C)								
Energy	–	0.705	0.888	0.902	0.831	–	0.785	0.033
Protein	–	0.970	0.975	0.965	0.899	–	0.925	0.014
Diet Digestibility – Atlantic salmon (15°C)								
Energy	–	–	–	–	–	–	–	–
Protein	0.772	0.775	–	0.862	0.799	0.809	0.816	0.013
Ingredient Digestibility – Atlantic salmon (15°C)								
Energy	–	–	–	–	–	–	–	–
Protein	–	0.713	–	0.902	0.787	0.793	0.891	0.032
Diet Digestibility – Atlantic salmon (6°C)								
Energy	0.792	0.751	0.846	0.826	0.761	0.766	0.773	0.013
Protein	0.836	0.840	0.881	0.879	0.838	0.849	0.843	0.007
Ingredient Digestibility – Atlantic salmon (6°C)								
Energy	–	0.722	0.946	0.923	0.679	0.723	0.697	0.049
Protein	–	0.911	0.968	1.077	0.705	0.848	0.794	0.054

Table 20.4 Summary of cross-correlations between each of the studies for diet and ingredient digestibilities of nitrogen and energy.

	Nitrogen			Energy		
Diet digestibilities	**RT**	**AS6**	**AS15**	**RT**	**AS6**	**AS15**
RT	–	–	–	–	–	–
AS6	0.978	–	–	0.743	–	–
AS15	0.877	0.883	–	–	–	–
Ingredient digestibilities						
RT	–	–	–	–	–	–
AS6	0.732	–	–	0.850	–	–
AS15	0.007	0.092	–	–	–	–

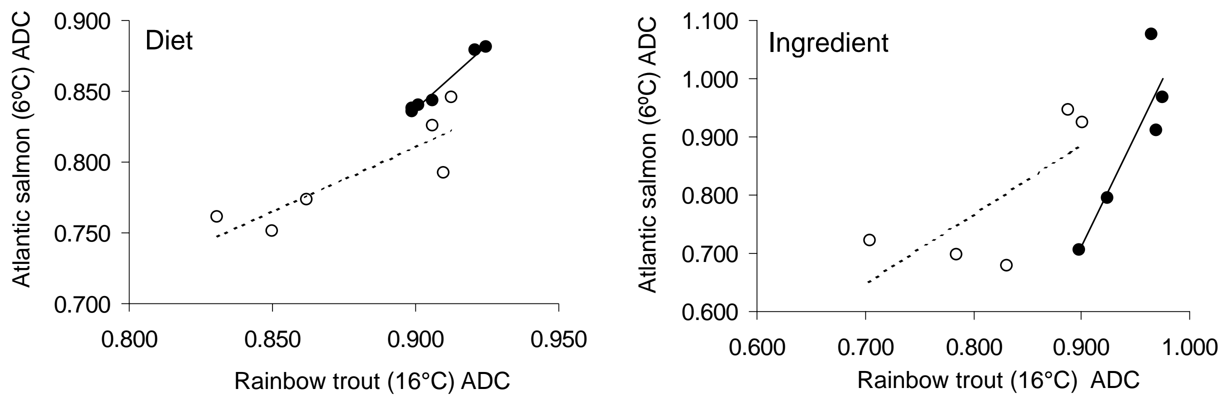

Figure 20.1 Correlations among diet (A) and ingredient (B) digestibilities of the same diets when fed to either Atlantic salmon at 6°C or rainbow trout at 16°C. Shown are the nitrogen (●), energy (o) digestibilities. Equation for regression functions are: diet nitrogen digestibilities y = 1.801x - 0.783, R2 = 0.978 and diet energy digestibilities y = 0.915x - 0.012, R2 = 0.743. Ingredient nitrogen digestibilities y = 4.857x - 3.730, R2 = 0.732 and ingredient energy digestibilities y = 1.231x - 0.186, R2 = 0.850.

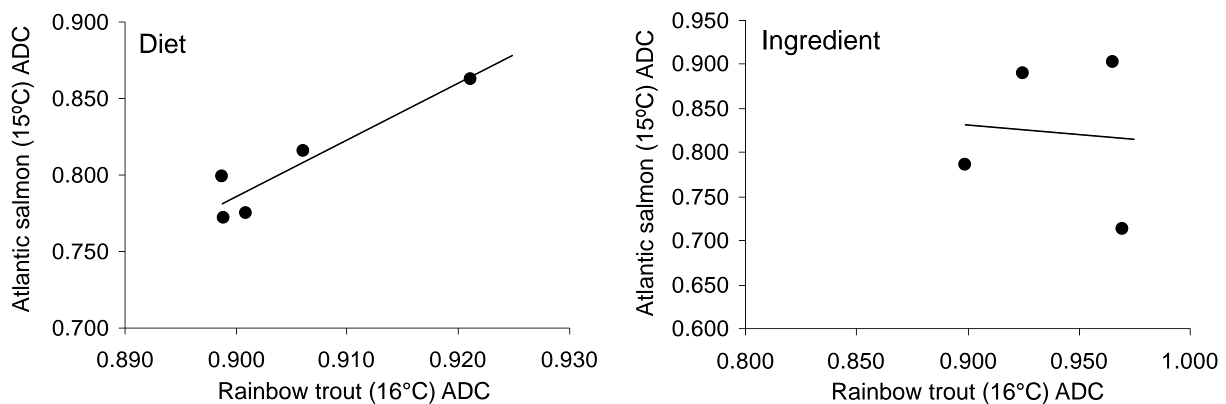

Figure 20.2 Correlations among diet (A) and ingredient (B) digestibilities of the same diets when fed to either Atlantic salmon at 15°C or rainbow trout at 16°C. Shown are the nitrogen (●) digestibilities. Equation for regression functions are: diet nitrogen digestibilities y = 3.640x - 2.535, R2 = 0.877. Ingredient nitrogen digestibilities y = 0.218x + 1.028, R2 = 0.007.

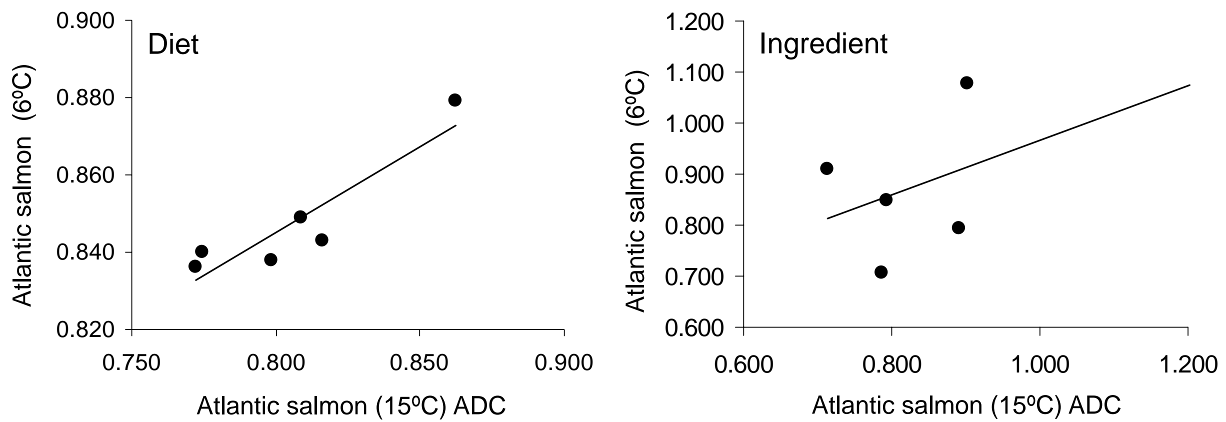

Figure 20.3 Correlations among diet (A) and ingredient (B) digestibilities of the same diets when fed to either Atlantic salmon at 15°C or Atlantic salmon at 6°C. Shown are the nitrogen (●) digestibilities. Equation for regression functions are: diet nitrogen digestibilities y = 0.444x + 0.490, R^2 = 0.883. Ingredient nitrogen digestibilities y = 0.535x + 0.430, R^2 = 0.092.

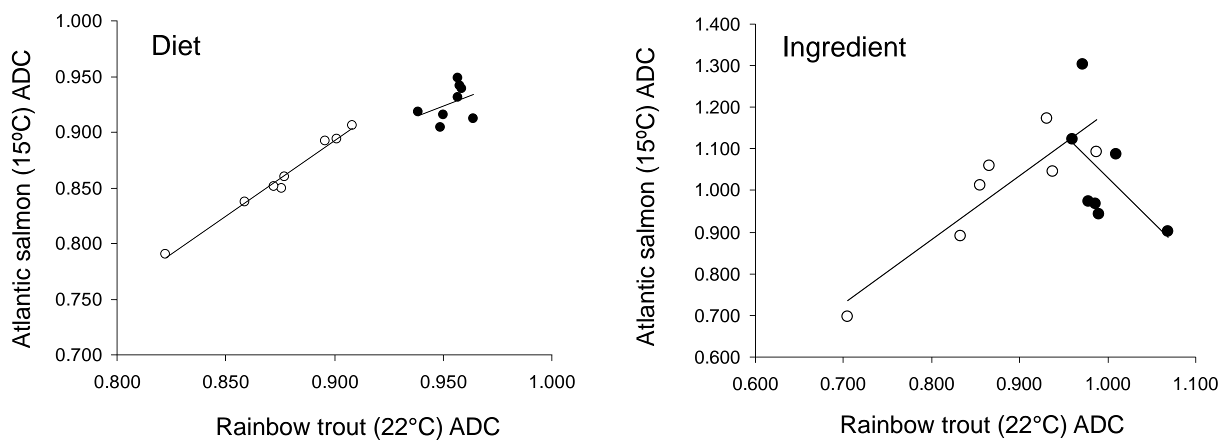

Figure 20.4 Data reproduced from Glencross et al. (2004b). Correlations among diet (A) and ingredient (B) digestibilities of the same diets when fed to either Atlantic salmon at 14°C or Rainbow trout at 15°C. Shown are the nitrogen (●), energy () digestibilities. Equation for regression functions are: diet nitrogen digestibilities: y = 0.7619x + 0.1996, R^2 = 0.1406; energy digestibilities: y = 1.354x - 0.3263, R^2 = 0.9845. Ingredient nitrogen digestibilities y = -2.0838x + 3.1163, R^2 = 0.2931, energy digestibilities: y = 1.5431x - 0.3528, R^2 = 0.8131.

21.0 Evaluation of the variability in the apparent digestible value of *Lupinus angustifolius* and *L. luteus* ingredients to Atlantic salmon, *Salmo salar*

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Abstract

The apparent digestibility of nutrients from kernel meals made from two narrow-leafed lupin (*Lupinus angustifolius*) and two yellow lupin (*L. luteus*) varieties were compared. Two additional ingredients, a *L. luteus* protein concentrate and a soybean reference, were also included. The ingredients were added to a basal fish meal mash at 30% and the diets extruded. Each diet was fed to three groups, one in each of three time-blocks, of Atlantic salmon (500 g) kept in 2000 l of seawater at 15°C. After 8 days the salmon were stripped of faeces and apparent digestibility calculated. There was no significant ($P > 0.2$) difference between ingredient apparent digestibility for crude lipid. The reference diet showed a low crude protein digestibility in one time period, when these data were removed there were significant differences between ingredient apparent digestibility for crude protein. Soybean and *L. angustifolius* cv. Myallie (MKM) had significantly lower AD for crude protein than *L. luteus* cv. Wodjil kernel meal (LKM) and protein concentrate (LPC). A *L. angustifolius* cv. Belara kernel meal (BKM) was not significantly different to any of the other ingredients. A second experiment aimed to determine the apparent digestibility of several varieties of *Lupinus angustifolius* kernel meals fed to seawater Atlantic salmon (*Salmo salar* L.). Faecal samples were stripped after 10 days on 5 experimental feeds containing 70% of a reference diet (REF) and 30% of either *L. angustifolius* (cv. Gungarru) kernel meal (GKM), *L. angustifolius* (cv. Mandelup) kernel meal (MaKM), *L. angustifolius* (cv. Myallie) kernel meal (MKM), *L. angustifolius* (cv. Tanjil) kernel meal (TKM), or *L. angustifolius* (cv. 2173M) kernel meal (2173KM). The apparent digestibility for crude protein was significantly higher for GKM and MKM than for MAKM. Gross energy, crude lipid and phosphorus digestibility were not different between ingredients. Ingredient crude protein digestibility was broadly similar to other similar studies.

21.1 Introduction

The importance of replacing fish meal and the potential of plant proteins has been well documented previously (Hardy, 1996; Carter, 2006; this volume). The use of lupins (*Lupinus* spp.) in aquafeeds for salmonids has received some attention in the literature, most information concerns rainbow trout (*Oncorhynchus mykiss*) and is more limited for Atlantic salmon (*Salmo salar*) (Carter and Hauler, 2000; Bransden et al., 2001). Atlantic salmon parr performed equally well when fed extruded feeds containing 25% lupin (*L. angustifolius*), field pea or soybean, lower growth efficiency at 33% inclusion was due to higher feed intake on the lupin feed (Carter and Hauler, 2000). Similarly, lupin kernel meal (*L. angustifolius*) added at 40% or at 20% in combination with feather meal did not affect growth performance or immune function of parr compared to a fish meal based control feed (Bransden et al., 2001). Digestibility of lupin (*L. angustifolius*) kernel meal, protein concentrate and a protein isolate were broadly similar for each ingredient between Atlantic salmon parr and rainbow trout (Glencross et al., 2004). Protein digestibility was uniformly high for all the lupin ingredients where as energy digestibility

increased as the proportion of protein increased from kernel to concentrate to isolate.

This experiment aimed to determine the apparent digestibility of a reference soybean meal, three lupin kernel meals and a lupin protein concentrate in seawater Atlantic salmon held at a "normal" summer water temperature of 15°C. Kernel meals from two varieties each of *L. angustifolius* and *L. luteus*, and the same diets were fed to rainbow trout (Chapter 5) and to Atlantic salmon at a lower water temperature of 5.6°C (Chapter 22; Refstie et al., 2006). An additional aim was to adopt standard approaches as used by the aquafeed industry for measuring apparent digestibility. This involved the development of procedures for transporting and holding seawater salmon in indoor recirculation facilities, stripping faecal samples and using time-blocks to allow replication. Atlantic salmon with an initial weight of about 500 g were held in six 2000 L recirculation systems, they were randomly assigned to a treatment group and stripped of faeces once after 8 days on a feed (Austreng, 1978; Percival et al., 1999). After being re-conditioned on a commercial feed for 6 to 7 days they were randomly reassigned to two further treatments and the process repeated.

In a second experiment the digestibilities of a range of *L. angustifolius* kernel meals were evaluated to examine the extent of variability in digestibility parameters for this raw material.

21.2 Materials and Methods

21.2.1 Experiment 1

21.2.1.1 Fish

All female pre-smolt Atlantic salmon were obtained from the Huon Aquaculture Company (Tasmania, Australia) over 3 weeks (farm weight estimate, 493 ± 42 g). Fish were held at the School of Aquaculture in six 2000-L Rathburn tanks that were each a self-contained partial recirculation system equipped with physical, biological and UV filtration. Water temperature was controlled at 15.0 ± 1.5°C and fish were exposed to ambient photoperiod. Water quality was maintained within recommended limits (Tarazona & Munoz 1995). A commercial salmon feed was hand fed 2-3 times per day for over an acclimation period of 4 to 6 weeks.

21.2.1.2 Diets

A reference mash was formulated and 5 experimental diets made to include 30% of each test ingredient (Table 21.1). The reference mash contained 0.1% Yttrium oxide as an inert digestibility marker. Ingredients tested were *L. luteus* protein concentrate (LPC), *L. luteus* (cv Wodjil) kernel meal (LKM), *L. angustifolius* (cv. Belara) kernel meal (BKM), *L. angustifolius* (cv. Myallie) kernel meal (MKM) and soybean meal (SBM). The dry ingredients were milled to 600 µm, mixed, extruded using a Wenger X185, and coated in oil (Refstie et al., 2006).

21.2.1.3 Apparent Digestibility (AD)

At the start of the apparent digestibility experiment all diets were hand fed three times a day to appetite and feed intake estimated from the weight of pellets fed. The six diets were randomly allocated to one group in each of three time periods. Diets were fed for 7 days and the salmon stripped (Austreng 1978; Percival et al. 2001) on day 8 in the morning. In order to randomise the effects of previous diets the fish were re-mixed during re-allocation to tanks. Groups were fed the commercial feed for 6 to 7 days and then transferred to the experimental diet for a further 7 days. Following initial sampling salmon were reused twice to obtain triplicate samples for each

diet. Faecal samples were freeze dried pooled into one sample per tank and one sample per tank analysed for each of Yttrium (ICPv, Y total by method iMET1STICP), crude protein (from the determination of total Nitrogen by SFA, based on %N x 6.25), crude fat (hexane extraction), phosphorous (ICP-AES) and ash. All analysis was conducted by the Chemistry Centre (WA), Department of Industry and Resources, Perth, Western Australia. The apparent digestibility (AD) values for protein, lipid and phosphorus were calculated using the standard formula: AD (%) = 100 – [100(%Idiet / %Ifaeces) X (%Nfaeces / %Ndiet)] (Maynard and Loosli, 1969), where I is the inert marker (Y_2O_3) and N the nutrient.

21.2.2 Experiment 2

21.2.2.1 Fish

Mixed-sex, diploid, very-late-spring (January) Atlantic salmon smolt were obtained from Mountain Stream Fishery (Targa, Tasmania, Australia) (farm weight estimate, 180 g). Salmon were held at the School of Aquaculture in six 2000-L Rathbun tanks that were each a self-contained partial recirculation system equipped with physical, biological and UV filtration. Water temperature was controlled at 15.0 ± 1.5°C, salinity at 30 ± 2 ppt and fish were exposed to ambient photoperiod. Water quality was maintained within recommended limits (Tarazona & Munoz, 1995). The fish were acclimated to the systems that were then used to hold the fish for the experiments. During acclimation a commercial salmon feed was hand fed two times per day for 8 weeks.

21.2.2.2 Diets

A reference mash was formulated and 5 experimental diets made to include 30% of each test ingredient. The reference mash contained 0.1% yttrium oxide as an inert digestibility marker. Five varieties of *Lupinus angustifolius* seed were collected from the Department of Agriculture (Western Australia) germplasm collection. Samples of the seed were then split using a small disc-mill and aspirated to separate hulls from kernels. A final manual clean of the kernels to remove any remaining hull material was also undertaken on each sample to ensure 100% purity of the kernel preparation. Each kernel sample was then milled using a Restsch rotor mill with a 750 µm screen to create a kernel flour. In addition to the lupin kernel flours, each of the other test ingredients used in this study was also thoroughly ground such that they passed through a 750 µm hammer mill screen. Ingredients tested were *L. angustifolius* (cv. Gungarru) kernel meal (GKM), *L. angustifolius* (cv. Mandelup) kernel meal (MaKM), *L. angustifolius* (cv. Myallie) kernel meal (MKM), *L. angustifolius* (cv. Tanjil) kernel meal (TKM), and *L. angustifolius* (cv. 2173M) kernel meal (2173KM). The dry ingredients were milled to 600 µm, mixed, Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient.

21.2.2.3 Apparent Digestibility (AD)

At the start of the apparent digestibility experiment all diets were hand fed two times per day at 0.6% body weight (BW). The six diets were randomly allocated to one group in each of three time periods. Diets were fed for 9 days and the salmon stripped (Austreng, 1978; Percival *et al.*, 2001) on the morning of day 10. In order to randomise the effects of previous diets the salmon were mixed during reallocation to tanks and fed the commercial diet for a further 18 days.

Following initial sampling salmon were reused twice to obtain triplicate samples for each diet.

Faecal samples from individual fish of known wet weight were pooled into 3 samples per tank and freeze dried. Chemical analyses for yttrium and phosphorus (ICP-OES), crude protein (%N x 6.25, elemental analysis), crude lipid (Soxhlet) and gross energy (bomb calorimeter) were determined from 3 faecal samples per tank, where the faecal sample was insufficient to do this it was pooled into 1 sample per tank.

The apparent digestibility (AD) values for protein, lipid and phosphorus were calculated using the standard formula: AD (%) = 100 –(Maynard and Loosli, 1969), where I is the inert marker (Y_2O_3) and N the nutrient. Apparent digestibility was calculated for each ingredient as AD_I (%) = (N_{test} x AD_{test} - 0.7 x N_{ref} x AD_{ref})/ (0.3 x N_I) (Sugiura et al., 1998) where AD_{ref} and AD_{test} were the apparent nitrogen digestibility of the reference and test diets, respectively, and N_{test}, N_{ref} and N_I the nutrient content of the reference diet, test diet and the ingredient, respectively. One-way ANOVA (n = 3) followed by a Tukey multiple comparison test were used to identify statistically significant differences between diets and ingredients.

21.3 Results and Discussion

21.3.1 Experiment 1

Seawater Atlantic salmon were successfully transferred to the School of Aquaculture, where they were acclimated and then maintained in 2000 L tanks until the experiment started. These fish were from commercial sea-cages and took time to acclimate to the experimental system. Initial feed intake was relatively low and the fish took between 2 and 3 weeks to reach a mean of 1% body weight day-1. When possible, future experiments should use salmon acclimated to tanks, grown on site at the Aquaculture Centre. The fish consumed the experimental feeds containing 30% lupin based protein sources. The mean (± SEM) weight of fish sampled in periods 1 to 3 was 597 ± 22 g, 611 ± 14 g and 634 ± 8 g, respectively, and there were no significant differences between weights in the 3 periods (F = 1.85; P = 0.20). Similarly there were no differences in the weights of fish sampled for the diets (Figure 21.1).

The AD values for crude protein, crude lipid and phosphorous from each period are detailed in Table 2. There was some variation between diet digestibility between the different time periods but this could not be analysed statistically due to there being one tank sample per time period. The variation between the digestibilities for REF was assessed further; crude protein was 5% different (lower) for period 2 than for the other two periods whereas for crude lipid all three values were within 5% of each other. In contrast, all three values for phosphorus were more than 5% different. There was a lack of resolution in the statistical comparison between the ingredients for crude protein and phosphorous (Table 21.3). This was as a consequence of the variation in the reference diet digestibility and the small differences between AD values of the REF and experiment diets. For crude protein (and phosphorous) the P value was marginal at 6%, when set at 10% there was a significant difference in crude protein digestibility between the SBM and LPC ingredients. There were no other significant differences. An alternate approach was to remove the crude protein data from period 2 due to the REF diet values being more than 5% different. When this was done there were significant differences between several ingredients (Figure 21.2). SBM and MKM had significantly lower AD for crude protein than LKM and LPC, BKM was not significantly different to any of the other ingredients. Thus, the *L. angustifolius* kernel meals had a lower crude protein AD than the *L. luteus* kernel meal although the difference was only significant between the Myallie cultivar. There was no

difference between the *L. luteus* kernel meal and the protein concentrate, a similar result to that observed between *L. angustifolius* kernel meal and protein concentrate (Glencross *et al*. 2004). The crude lipid AD for the REF diet was less varied and there were no significant differences between ingredients at P equal to 50% and provided a stronger indication that crude lipid digestibility was not significantly different between ingredients. This was explained because the majority of lipid came from fish meal and fish oil and not from the test ingredients, and it also suggested that the test ingredients did not have any further effect on lipid digestibility within the experimental diets.

The same ingredients and diets were used in a study on Atlantic salmon which investigated apparent digestibility in slightly smaller Atlantic salmon (176 g) and at a considerably lower water temperature of 5.6°C (Refstie *et al*., 2006). There were fewer significant differences between ingredient crude protein digestibility values and only the lupin protein concentrate had a significantly higher digestibility. The value was 88% compared with 86% from the current experiment. The major difference between the Refstie *et al*. (2006) and the current experiment were for soybean, 84 compared with 70%, and MKM, 84 compared with 72%, respectively. Refstie *et al*. (2006) found lipid digestibility was similar for the lupin ingredients but significantly lower for soybean, this was not found in the current experiment which didn't find any differences between the ingredients. Lupin lipid digestibility was 7 to 12% points higher in the current experiment and probably explained by the higher temperature.

21.3.2 Experiment 2

Although lupins are generally well utilised by salmonids it is important to identify factors that influence utilisation, even by small amounts, in order to identify and develop the best range of lupin products for use in salmonid feeds. The current research focused on digestibility in seawater Atlantic salmon held at a normal Tasmanian summer temperature and compared five varieties milled as kernel meals from the same lupin species (*L. angustifolius*). There were some differences between lupin varieties, the only significant being between AD crude protein where as there were no significance differences in AD crude lipid, phosphorus or gross energy. For AD crude protein GKM, MKM and TKM included at 30% were significantly better digested than MAKM.

21.3.2.1 Apparent digestibility

Crude protein digestibility varied between ingredients, MKM, GKM and TKM had significantly higher AD than MAKM (Table 21.5). There were no significant differences between the six diets, including REF, at each of the time periods. Compared with the diets faecal samples contained proportionately very small amounts of lipid. This meant that sub-samples had to be combined for each time period. There were no differences in the AD lipid between the ingredients. Dietary phosphorus was between 1.4 and 1.8% (Table 21.4) and diet phosphorus AD ranged between 33 and 47% (Appendix 3). In period 2, MAKM dietary phosphorus AD was significantly higher than for MKM and TKM, there were no other differences in any period. Ingredient phosphorus AD were lower than dietary phosphorus AD, they ranged between 5 and 16% and were not different between the ingredients (Table 21.5). Gross energy diet AD values showed outliers for GKM and MKM during period 3 so the data were removed prior to analysis. There was large variation between samples and there were no significant differences in AD gross energy between the ingredients (Table 21.5). AD energy is probably the least reliable measure in the present research.

In terms of crude protein one of the lupin ingredients was the same as used in a Norwegian AD study using seawater Atlantic salmon of around 176 g but held at a significantly lower water

temperature of 5.6°C (Refstie *et al.*, 2006). The Myallie kernel meal (MKM) had similar AD crude protein of 80 and 84% in the present and Norwegian research, respectively. Similarly, fish meal had AD crude protein of 80 and 84% in the present and Norwegian research, respectively. In terms of crude protein the AD values from the present research were slightly lower than the Norwegian study but importantly, the similarity between the two ingredients within each study was shown.

21.3.2.2 Atlantic salmon

There was no difference in mean wet weight of fish between diet treatments. The experimental design where by each ingredient was tested in each period removed weight as a potential variable in relation to change over time. Mean (± SE) wet weight increased over the experiment and was significantly ($P < 0.001$) different for each of the periods: 333.7 ± 2.8 g, 367.1 ± 5.1 g and 406.5 ± 7.7 g for periods 1,2 and 3, respectively. The moderate but increased weight suggested the fish were relatively well acclimated to the experimental regime.

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Table 21.1 Formulation of experimental Atlantic salmon feeds containing different plant protein ingredients.

	REF	LKM	LPC	BKM	MKM	SBM
Ingredient composition (g/kg)						
Fish meal	700	490	490	490	490	490
Fish oil	150	105	105	105	105	105
Wheat flour	144	100.8	100.8	100.8	100.8	100.8
Pre-mix vitamins	5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1	0.7	0.7	0.7	0.7	0.7
Plant protein		300	300	300	300	300
Chemical composition (g/kg DM)						
Crude protein	453	478	538	436	440	463
Crude lipid	222	175	194	184	194	159
NFEb	197	268	162	276	273	267
Ash	128	79	106	104	93	111

Diets: REF, reference; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.
[b]Calculated by subtracting crude protein, crude lipid and ash. Assumes crude protein is 6.25 X N.

Table 21.2 Apparent digestibility of reference and experimental diets at different time periods.

	REF	LKM	LPC	BKM	MKM	SBM
Crude protein						
Period 1	78.07	80.31	84.57	80.40	78.67	78.96
Period 2	73.45	83.19	87.88	79.55	81.01	75.67
Period 3	80.16	81.34	86.26	82.66	79.92	77.73
Mean	77.23	81.61	86.24	80.87	79.87	77.45
SEM	1.98	0.84	0.96	0.93	0.68	0.96
Crude lipid						
Period 1	95.21	93.86	94.45	94.80	98.59	95.42
Period 2	94.69	96.53	98.10	96.57	99.81	94.42
Period 3	91.69	97.44	93.09	94.33	93.28	92.86
Mean	93.86	95.94	95.21	95.23	97.23	94.23
SEM	1.10	1.07	1.50	0.68	2.00	0.74
Phosphorus						
Period 1	28.91	36.02	32.61	41.53	36.19	29.49
Period 2	20.18	29.62	31.99	27.73	11.66	22.62
Period 3	13.32	26.08	18.11	38.23	24.95	32.34
Mean	20.80	30.57	27.57	35.83	24.27	28.15
SEM	4.51	2.91	4.73	4.16	7.09	2.88

Diets:REF, reference; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.

Table 21.3 Apparent digestibility of ingredients fed to seawater Atlantic salmon using data from 3 time periods.

	LKM	LPC	BKM	MKM	SBM	P
Crude Protein	89.06	90.24	79.25	78.67	71.32	0.06
	5.99	4.29	2.39	6.26	2.51	NS
Crude Lipid	95.94	95.21	95.23	97.23	94.23	0.50
	1.07	1.50	0.68	2.00	0.75	NS
Phosphorus	103.00	29.10	184.80	61.40	78.90	0.06
	7.19	7.36	48.38	64.00	37.70	NS

Ingredients: LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.

Mean ± SEM (n = 3)

Table 21.4. Formulation of experimental Atlantic salmon feeds containing different plant protein ingredients.

	REF	GKM	MAKM	MKM	TKM	2173KM
Ingredient composition (g/kg)						
Fish meal	700	490	490	490	490	490
Fish oil	150	105	105	105	105	105
Wheat flour	144	100.8	100.8	100.8	100.8	100.8
Pre-mix vitamins	5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1	0.7	0.7	0.7	0.7	0.7
Plant protein ingredient		300	300	300	300	300
Chemical composition (g/kg DM)						
Dry matter (g/kg)	972	982	981	959	968	964
Crude protein	509	505	494	504	507	501
Crude lipid	212	155	163	160	162	163
NFE[1]	161	244	253	243	240	248
Ash	118	96	90	93	91	88
Phosphorous	18.4	14.8	14.5	13.9	14.4	14.4
Energy (MJ/kg DM)	22.4	21.8	22.0	21.5	22.1	21.9

Diets: REF, reference; GKM, *L. angustifolius* (cv. Gungurru); MAKM, *L. angustifolius* (cv. Mandelup); MKM, *L. angustifolius* (cv. Myallie); TKM, *L. angustifolius* (cv. Tanjil); 2173KM, *L. angustifolius* (Coromup(W2173))

[1] Nitrogen free extractives (NFE) calculated by subtracting crude protein, crude lipid and ash.

Table 21.5 Apparent digestibility (%) of ingredients fed to seawater Atlantic salmon.

	GKM	MAKM	MKM	TKM	2173KM	P
Crude protein	78.41[a]	60.31[b]	79.85[a]	77.62[a]	71.42[ab]	0.01
	3.28	3.43	5.71	3.08	3.55	
Crude lipid	80.81	78.86	83.11	81.18	79.22	0.59
	2.84	2.54	1.12	1.48	1.41	NS
Phosphorus	10.81	16.18	5.48	6.87	13.18	0.21
	2.74	3.05	4.26	3.65	3.89	NS
Gross energy	59.94	68.27	65.49	75.31	71.45	0.85
	5.38	7.97	11.29	9.75	8.46	NS

Ingredients: GKM, *L. angustifolius* (cv. Gungurru); MAKM, *L. angustifolius* (cv. Mandelup); MKM, *L. angustifolius* (cv. Myallie); TKM, *L. angustifolius* (cv. Tanjil); 2173KM, *L. angustifolius* (Coromup (W2173))

Mean ± SE (n = 3), means with different superscript were significantly different using Tukey Multiple comparison. Multiple comparison did not identify differences between ingredients for Gross energy.

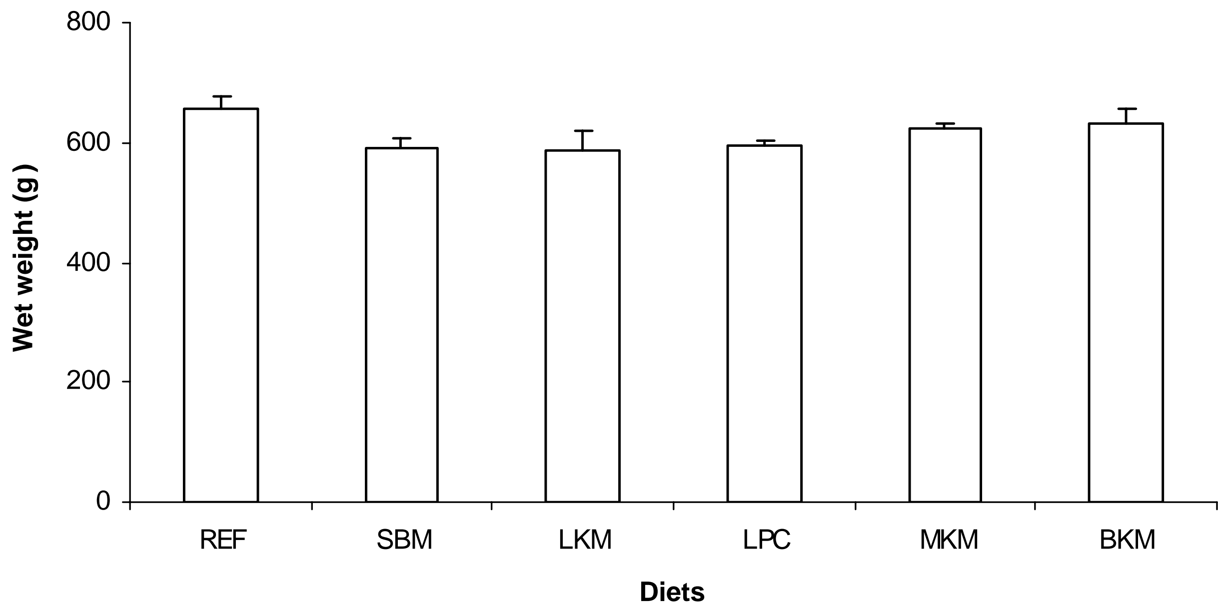

Figure 21.1 Mean (± SEM) wet weight of Atlantic salmon sampled for faeces when fed one of six diets (n = 3; P = 0.20). Diets: REF, reference; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.

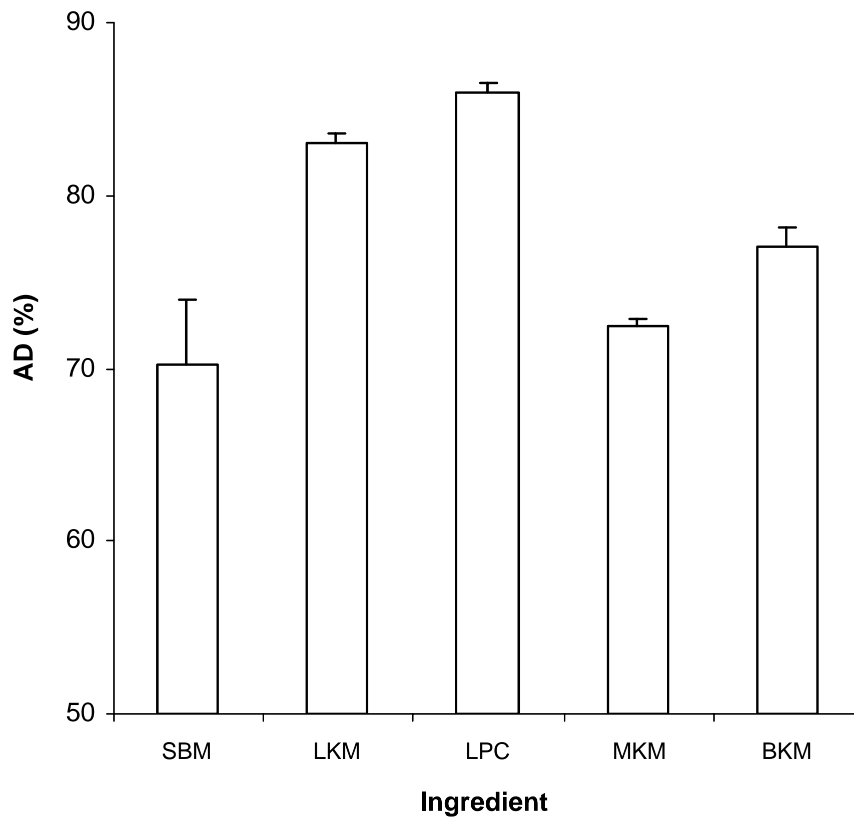

Figure 21.2 Mean (± SEM) apparent digestibility for crude protein for Atlantic salmon fed one of five ingredients. Ingredients: SBM, soybean meal; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal. (n = 2; P = 0.007. Multiple comparison LPC^{a}, LKM^{a}, BKM^{ab}, MKM^{b}, SBM^{b}).

22.0 Gastrointestinal evacuation rate in seawater Atlantic salmon (*Salmo salar*) fed diets containing fish meal, soybean meal, lupin kernel meals and lupin protein concentrates

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Abstract

The experiment aimed to assess the effect of plant proteins on the gastrointestinal evacuation rate (GIER) of Atlantic salmon (*Salmo salar* L.) smolts (144.2 ± 5.8 g) held at 15°C. Ingredients tested were *L. luteus* protein concentrate (LPC), *L. luteus* (cv Wodjil) kernel meal (LKM), *L. angustifolius* (cv. Belara) kernel meal (BKM), *L. angustifolius* (cv. Myallie) kernel meal (MKM), soybean meal (SBM). A reference mash that included an inert marker (0.1%) was formulated and 5 experimental diets made to include 30% of each plant protein ingredient. Two sets of each diet containing either 0.1% Yttrium oxide or 0.1% Ytterbium oxide as inert markers were made. Calculation of the GIER was based on the replacement of one marker with the second marker in faeces collected after the markers were changed in the diets being fed. A model described by marker (%) = $(a - d) / ((1 + (T/c)^{-b}) + d)$ was used to derive values for the slope (parameter b) of an S-shaped curve and the time taken for the second marker to replace half the first marker (parameter c). In relation to the time taken for 50% replacement (parameter c), the ingredients were divided into two groups: the kernel meals (LKM, BKM, MKM) had values of 8.5 to 8.8 h compared with 10.2 to 11.4 h for the other ingredients (LPC, REF, SBM). Groupings in the slope value (parameter b) were less obvious although the kernel meals had the lowest values and LPC had the highest value, almost twice that of the lowest, MKM. The higher slope values indicated more of the gut contents tended to be evacuated together whereas the lower slope values indicated a more gradual evacuation. Apparent digestibility for dietary nitrogen was positively correlated with slope ($r = 0.829$; $P < 0.01$; $n = 6$). Overall, the analysis suggested that more of the lupin kernel meals were evacuated sooner but in a more gradual manner. In comparison, the other meals, particularly LPC, remained in the gastrointestinal tract longer but were then evacuated more rapidly in a consolidated mass.

22.1 Introduction

Gastrointestinal evacuation is a key process in feeding and digestion, gastric evacuation has a strong influence on feed intake and return of appetite, there may also be relationships between digestibility and the movement of materials through the stomach and intestine (Talbot, 1985). A range of abiotic and biotic factors influence both gastric (GER) and gastrointestinal (GIER) evacuation rates in fish, water temperature is the main abiotic factor whilst species, life stage and body weight are key endogenous biotic factors. Factors more specific to nutrition include pellet characteristics; feed composition such as the energy density or the amount of indigestible material; and the magnitude of feed intake, such as the number of meals and daily ration (Jobling, 1987). Fish also adapt to different nutritional regimes. For example, after 10 weeks rainbow trout (*Oncorhynchus mykiss* Walbaum) fed a wet diet made from herring mash had larger stomachs than those fed a dry diet (Ruononen and Grove, 1996). A variety of methods

have been used to measure GER and include serial slaughter and serial flushing of stomach contents. Neither of these methods are useful for measuring GEIR, this can be measured from the progress of labelled compounds along the gastrointestinal tract measured from either serial slaughter or their appearance in the faeces (Talbot, 1985). Storebakken et al. (1999) measured the replacement of one inert marker in the faeces by another and showed that in Atlantic salmon (*Salmo salar* L.) soybean may slow down GIER compared with fish meal or bacterial meal. The method is advantageous because the fish are not disturbed during faecal collection and assumed to perform feeding and digestive behaviours normally. However, the method does not appear to have received much attention in fish nutrition but offers potential for comparing the effects of different ingredients.

Lupin (*Lupinus* spp.) products offer potential as protein sources for use in aquafeeds, particularly to replace fish meal in salmonid diets (Burel et al., 1998; Carter and Hauler, 2000; Glencross et al., 2004). However, lupins contain relatively high amounts of soluble and insoluble non-starch polysaccharides and oligosaccharides that may have anti-nutritional effects on animals including fish (van Barneveld, 1999; Glencross et al., 2003). Digestion may be affected through their interference in the digestion of other nutrients such as amino acids (Glencross et al., 2003). Due to the lupin carbohydrates sticky droppings occur in poultry fed high levels of lupins and have the potential to change GIER (Rothmaier and Kirchgessner, 1994). Consequently, the current experiment aimed to determine whether GIER of seawater Atlantic salmon was influenced by the inclusion of different lupin protein sources. Three lupin kernel meals and a lupin protein concentrate were used at a high dietary inclusion of 30% and compared with a reference fish meal diet and a soybean diet. The ingredients were the same as in Chapter 17 of this volume and by Refstie et al. (2006). Analysis of GIER was based on measuring the replacement of one inert marker with a second inert marker in the faeces collected after the markers were changed in the diet (Storebakken et al., 1999).

22.2 Materials and Methods

22.2.1 Fish

Atlantic salmon (*Salmo salar* L.) pre-smolt were obtained from Wayatinah Salmon Hatchery (SALTAS, Tasmania, Australia). One hundred and forty four fish were distributed between 12 300-l conical bottomed tanks at 12 fish per tank (144.2 ± 5.8 g) at the School of Aquaculture. The fish were acclimated to saltwater over 4 days then hand fed a commercial salmon feed for 6 weeks. Fish were held in a partial recirculation system and water treated with physical, biological and UV filtration. Water temperature was controlled at 15.0 ± 1.5°C and fish were exposed to controlled photoperiod, L:D 16:8h. Water quality (dissolved oxygen, pH, salinity, ammonia, nitrite, nitrate) was monitored regularly and maintained within recommended limits (Tarazona and Munoz, 1995).

22.2.2 Diets

A reference mash was formulated and 5 experimental diets made to include 30% of each test ingredient and 0.1% inert marker (Table 1). Two sets of reference mash containing either 0.1% yttrium oxide or 0.1% ytterbium oxide as inert markers were used to make extruded diets (Refstie et al., 2006). Ingredients tested were *L. luteus* protein concentrate (LPC), *L. luteus* (cv Wodjil) kernel meal (LKM), *L. angustifolius* (cv. Belara) kernel meal (BKM), *L. angustifolius* (cv. Myallie) kernel meal (MKM), soybean meal (SBM). The extruded diets were re-pelleted to

a 4.5 mm diameter size using a California laboratory pellet mill (CL-2), dried at 35°C for 16 h and stored at below 4°C.

22.2.3 Gastrointestinal Evacuation Rate

The gastrointestinal evacuation rate (GIER) experiment was based the replacement of one marker with a second marker in the same feed and faecal collection before and after the markers were changed (Storebakken et al., 1999). Fish were held in 300-l conical bottomed tanks fitted with Guelph type faecal collectors (Carter and Hauler, 2000). Ytterbium-labelled feed was fed via belt-feeders twice per day for 8 days, on day 9 the feed was replaced by the yttrium-labelled feed and faecal samples taken over the following 28 h at 0, 4, 8, 12, 16, 20, 24 and 28 h.

For the analysis of yttrium and ytterbium freeze-dried samples of approximately 200 mg DM were weighed to the nearest mg and subjected to wet-decomposition at 100°C with 5 ml of 16 M nitric acid (Aristar Grade) followed by the addition of 5 ml of 30% w/w hydrogen peroxide (AnalR grade) and heated to 100°C. After decomposition the samples were made up to a volume of 25 ml with distilled water. Following a further x50 dilution the samples were analysed using inductively coupled plasma optical emission spectrophotometry (Thermo Jarrell-Ash IRIS Axial ICP-OES). Blank samples, containing only the decomposition acid, were included (Ward et al., 2005).

GIER was calculated according to Storebakken et al. (1999) and expressed as the percent of the two markers that was accounted for by the second marker where M2 (%) = 100 x (M2 / M1 + M2). Regression analysis was conducted using Sigmaplot according to the model: M2 (%) = (Max – Min) / ((1 + $(T/T_{0.5})^{-b}$) + Min) where Max and Min are the upper and lower asymptotes, b the slope (Kinetic order), T the time in hours and $T_{0.5}$ the time at which half the marker was M2. Spearman rank correlation values were calculated using SPSS.

22.2.4 Apparent digestibility

The fish were too small to use stripping to obtain faecal samples and the Guelph-type settlement collectors used to collect faeces overnight following the GIER collection period (Carter and Hauler, 2000). Faecal samples were freeze dried, pooled into one sample per tank and one sample per tank analysed for each of yttrium (ICPv, Y total by method iMET1STICP) and nitrogen (Thermo Finnigan EA 1112 Series Flash Elemental Analyser). The diet apparent digestibility (AD) value for nitrogen was calculated using the standard formula: AD (%) = 100 – [100($\%I_{diet} / \%I_{faeces}$) X ($\%N_{faeces} / \%N_{diet}$)] (Maynard and Loosli, 1969), where I is the inert marker (Y_2O_3) and N nitrogen. Apparent digestibility for nitrogen for each ingredient was calculated as AD_I (%) = (N_{test} x AD_{test} - 0.7 x N_{ref} x AD_{ref})/ (0.3 x N_I) (Sugiura *et al.*, 1998) where AD_{ref} and AD_{test} were the apparent nitrogen digestibility of the reference and test diets, respectively, and N_{test}, N_{ref} and N_I the nutrient content of the reference diet, test diet and the ingredient, respectively. There was only sufficient faecal material for analysis of the marker and nitrogen.

22.3 Results

Apparent digestibility of nitrogen was not significantly different between the diets but there were significant differences between the ingredients, LKM was significantly higher than BKM and MKM (Table 1). The GIER model described the data well as shown by high R^2 values of over 94% (Table 2). Curves were not compared statistically but there were apparent differences in the pattern of GIE between the ingredients (Fig. 1). The measured proportion of marker

2 at 28 h was over 95% for MKM (99%), LPC (98%), REF (97%), LKM (97%) and BKM (96%). After 28 h the model predicted slightly different rates so that it was nearly complete for REF and LKM with a plateau (maximum) at over 97% where as the maximum predicted for other ingredients ranged between 88% for SBM to 93% for MKM. The low predicted SBM maximum was due to a large difference between the two replicate measurements at 28 h (77 and 97%). Ingredient $T_{0.5}$ values appeared to separate into two groups, the kernel meals (LKM, BKM, MKM) had values of 8.5 to 8.8 h compared with 10.2 to 11.4 h for the other ingredients (LPC, REF, SBM). Groupings in the slope values were less obvious although the kernel meals had lower values and the LPC had a value that was almost twice that of MKM (Table 2). Higher slope values indicated faster evacuation of the majority of the gut contents whereas a lower slope indicated a more gradual evacuation. There was a significant correlation between $T_{0.5}$ and slope ($r = 0.829$; $P < 0.01$; $n = 6$) and showed the relationship between the two parameters of the model.

The influence of diet composition on model parameters was indicated by the significant negative correlation between NFE and slope ($r = -0.771$; $P < 0.05$; $n = 6$) and a positive correlation between ash and $T_{0.5}$ ($r = 0.886$; $P < 0.01$; $n = 6$). This showed that as dietary carbohydrate content increased the slope was steeper whereas increased ash was correlated with a longer $T_{0.5}$. Apparent digestibility for dietary nitrogen was positively correlated with slope ($r = 0.829$; $P < 0.01$; $n = 6$) but not with $T_{0.5}$. Ingredient AD was not correlated with either slope or $T_{0.5}$.

22.4 Discussion

Analysis of the gastrointestinal evacuation rate (GIER) suggested that a greater proportion of the lupin kernel meals were evacuated sooner but in a more gradual manner compared to the other meals, particularly LPC. Thus, the protein concentrate remained in the gastrointestinal tract longer but was then evacuated more rapidly in a more consolidated mass. Similarly, soybean had the longest $T_{0.5}$ and the second highest slope value. The high content of carbohydrate fractions, such as NSP and oligosaccharides, in the kernel meals may partly explain differences in the patterns of evacuation. Compared to the other three diets, for the kernel meals, GIER was faster as judged by lower $T_{0.5}$ and more continuous as indicated by the lower slope values. The influence of kernel carbohydrates was supported by the negative correlation between dietary NFE content and slope, this suggested that the greater the content of NFE the more continuous evacuation was.

22.4.1 Gastrointestinal evacuation rate

Lupin oligosaccharides reduced nitrogen digestibility in rainbow trout (Glencross et al., 2003). Oligosaccharide content would have been highest in the kernel meal diets used the present experiment. It is therefore of interest that there was a correlation between model parameters and dietary AD nitrogen values. Higher AD nitrogen was correlated with higher slope values, ie with a more discrete evacuation. It is not possible to determine cause and effect and determine whether the pattern of GIE changed in response to the ingredients or in response to digestion and release of nutrients from the ingredients. Neither the diet nor ingredient AD values from the larger Atlantic salmon detailed in Chapter 17 and fed the same ingredients correlated with model parameters.

The current study used 140 g Atlantic salmon at 15°C and can usefully be compared with the one other previous study (Storebakken et al., 1999), this used 150-200 g Atlantic salmon at 9°C. In the latter experiment fish meal had values of 7.5 and 18.2 h for the slope (parameter b) and

$T_{0.5}$ (parameter c), respectively. In the current experiment the values were 7.7 and 10.7 h, and suggested the slope was related more strongly to the ingredient whereas the over all speed of GIE was related more to the influence of water temperature on physiological rates. This was to some extent confirmed by comparing soybean between the two studies, $T_{0.5}$ was 11.4 h in the present study and lower than 19.8 h measured at 9°C. Interestingly, the values were 7 to 9% higher for soybean than for fish meal, perhaps indicating an ingredient component. The slope values for soybean from the 9 and 15°C experiments were 11.0 and 8.6 h, respectively; they were both larger than corresponding values for fish meal. The close similarity in the slope values of the kernel meals in the present study indicated testing the hypothesis that slope value relates more closely to the ingredient and slope relates to ingredient and temperature is worthy of further investigation. As noted above, the two parameters are also interrelated to some extent.

22.4.2 Lupins

In a parallel study, Refstie et al. (2006) fed similar ingredients and diets to similar sized (176 g) Atlantic salmon, a low temperature of 5.6°C was a significant difference between the studies. AD values were generally higher in the current study, probably due to using stripping by Refstie et al. (2006) rather than settlement used in the present study. In terms of differences the protein concentrates had higher AD nitrogen values than the kernel meals which were similar to soybean and fishmeal. In the current study the AD nitrogen for two kernel meals (BKM, MKM), fish meal and soybean were also similar. The main difference between the two studies was the LKM had a significantly higher AD nitrogen than the other two kernel meals, the reasons for this are not clear. Refstie et al. (2006) also investigated the effect of lupins on gut pathomorphology due to reports of soybeans causing changes to Atlantic salmon distal intestine (Rumsey et al., 1995). In rainbow trout the inclusion of *L. angustifolius* kernel meal from 0 to 50% did not cause any differences in the digestive physiology as measured by trypsin or amylase activity, pylorus size or villus height, nor where there any notable differences in the non-specific immune response (Farhangi and Carter, 2001). Lupins did not cause lesions to the distal intestine of Atlantic salmon but appeared to worsen gastric lesions that may have been caused initially by poor quality fish meal (Refstie et al., 2006). These effects were not different between three kernel meals and two protein concentrates so it seems unlikely that the differences in GIER reported in the current study would have contributed to them. Nevertheless it suggests that an investigation of gastric evacuation would be of interest and may highlight some differences between lupins and other ingredients.

22.5 References

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Table 22.1 Formulation of experimental Atlantic salmon diets containing different plant protein ingredients.

	REF	LKM	LPC	BKM	MKM	SBM
Ingredient composition (g/kg)						
Fish meal	700	490	490	490	490	490
Fish oil	150	105	105	105	105	105
Wheat flour	144	100.8	100.8	100.8	100.8	100.8
Pre-mix vitamins	5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide or Ytterbium oxide	1	0.7	0.7	0.7	0.7	0.7
Plant protein[1]		300	300	300	300	300
Chemical composition (g/kg DM)						
Crude protein	453	478	538	436	440	463
Crude lipid	222	175	194	184	194	159
NFE[2]	197	268	162	276	273	267
Ash	128	79	106	104	93	111
M1 (in diets with Yb_2O_3)	1.02	0.72	0.71	0.73	0.72	0.74
M2 (in diets with Y_2O_3)	0.97	0.69	0.76	0.77	0.76	0.72
Apparent digestibility (%)[3]						
Nitrogen (diets)	93.64	93.81	94.84	93.21	92.18	93.95
	0.36	0.26	0.31	0.09	1.39	0.17
Nitrogen (ingredients)	93.64	101.2	93.59	87.91	87.16	94.90
	0.36[ab]	0.81[b]	0.72[ab]	0.32[a]	4.85[a]	0.53[ab]

[1] Plant protein diets: REF, fish meal reference; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.

[2] Calculated by subtracting crude protein, crude lipid and ash.

[3] One-way ANOVA followed by Tukey multiple comparison (Diets F = 2.07, P = 0.20; Ingredients F = 6.02 , P = 0.025)

Table 22.2 Parameters for the model[a] describing gastrointestinal evacuation rates of diets containing plant proteins fed to Atlantic salmon.

Diet[b]	Min (%)	Max (%)	$T_{0.5}$ (h)	b	n	R^2	P
REF	0.12	97.09	10.68	7.67	16	99.5	<0.0001
	(1.41)	(1.89)	(0.16)	(0.68)			
LKM	0.60	97.80	8.55	6.46	16	99.8	<0.0001
	(1.05)	(1.36)	(0.09)	(0.48)			
LPC	1.10	92.60	10.17	10.72	15	98.0	<0.0001
	(3.16)	(3.96)	(0.48)	(2.54)			
BKM	2.72	91.92	8.82	6.54	16	94.0	<0.0001
	(5.20)	(6.76)	(0.51)	(2.27)			
MKM	4.97	93.53	8.48	5.60	15	96.9	<0.0001
	(3.82)	(5.36)	(0.37)	(1.52)			
SBM	4.57	88.52	11.43	8.59	15	95.8	<0.0001
	(4.04)	(5.77)	(0.48)	(4.17)			

[a] Model: $M2(\%) = (Max - Min) / ((1 + (T/T_{0.5})^{-b}) + Min)$

[b] Plant protein diets: REF, fish meal reference; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.

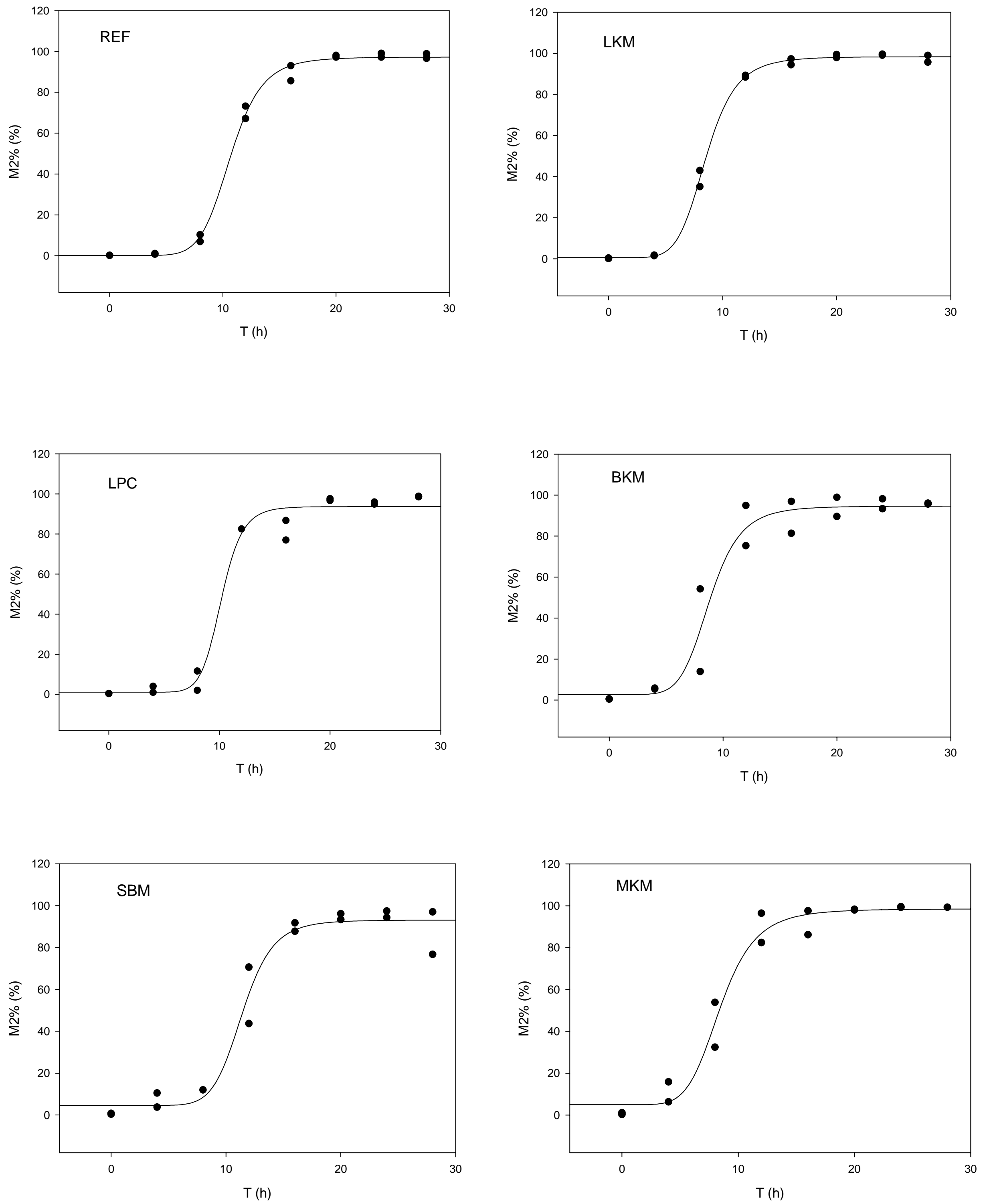

Figure 22.1 Gastrointestinal evacuation shown by the percent of marker 2 (M2) in the faeces of Atlantic salmon fed fish meal and plant proteins diets: REF, fish meal reference; LKM, *L. luteus* (cv Wodjil) kernel meal: LPC, *L. luteus* protein concentrate; BKM, *L. angustifolius* (cv. Belara) kernel meal; MKM, *L. angustifolius* (cv. Myallie) kernel meal; SBM, soybean meal.

23.0 Biological value to Atlantic salmon of lupin kernel meal compared with soybean at different inclusions and water temperatures

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Abstract

The experiment aimed to compare the biological value of a lupin kernel meal (*L. angustifolius* cv Coromup) with fish meal and with soybean at two temperatures and two inclusion levels. Inclusion levels of lupin and soybean were 15 and 25% at 14°C and 15% at 18°C. Diets were formulated to be isonitrogenous and isoenergetic on a gross compositional basis and to have a marginal crude protein content (40%). Inclusion of 15% reflected maximum industry inclusion rates for lupin where as 25% inclusion reflected a higher level in order to investigate whether performance changed at the higher level. The temperature of 14°C reflected an optimum summer temperature and was compared with an elevated summer temperature of 18°C, but one at which salmon would still be fed commercially. Following initial analysis a two-way ANOVA compared the effects of diet and temperature using a data set restricted to the 15% inclusion. There was no interaction between temperature and diet for any performance parameter analysed and the key results were: for change in weight both diet ($P = 0.009$) and temperature ($P = 0.001$) were significant factors, LM15 and 14°C showed significantly higher change in weight; weight specific feed intake was also significantly higher for LM15 but it was higher at 18°C. This meant growth efficiency was not different between diets but was lower at 18°C. Thus, in terms of growth performance LM15 appeared to be the better diet at both temperatures. However, exposure to plant meals as well as to high temperature, in addition to ingredient effects, contributed to moderate / severe morphological changes observed in the intestinal mucosa.

23.1 Introduction

Ingredient inclusion experiments are usually conducted under optimum conditions, including optimum temperature, in order to promote maximum feed intake and growth responses in the fish. Consequently, nutrition experiments are not usually conducted under more extreme conditions (Carter et al., 2005), although this now changing in relation to climate change and, in particular, to improve aquaculture practice in regions where species have traditionally been farmed outside of the optimum range such as Atlantic salmon in Tasmania (Carter et al., 2003; Miller et al., 2006).

A growing body of research confirms the early promise of lupins as nutritious ingredients in Atlantic salmon aquafeeds (Carter, 1998; Carter & Hauler, 2000). Atlantic salmon parr fed extruded feeds containing 25 and 33% of three different plant meals grew equally well on soybean meal, field pea and lupin protein concentrates (Carter & Hauler, 2000). However, it has also been suggested that lupins may affect the gastrointestinal tract of salmonids (Farhangi & Carter, 2001; Refstie et al., 2006). Although, increasing dietary lupin from zero to fifty percent had no effect on trypsin activity, amylase activity or villus height in the proximal intestine of rainbow trout (Farhangi & Carter, 2001). However, a non significant decrease in villus height suggested that it would be worth investigating gastrointestinal tract morphology in further

studies. Ulcer-like lesions in the stomach of seawater Atlantic salmon were found in fish fed fish meal control and soy bean diets but were considered worse in fish fed lupins (Refstie et al., 2006). In contrast, there was no evidence of the enteritis-like pathomorphological changes to the intestine often associated with soybean inclusion (Van den Ingh et al., 1991; Refstie et al., 2006). An aim of the present experiment was to investigate the gastrointestinal tract.

The experiment aimed to compare the biological value of a lupin kernel meal (*L. angustifolius* cv Coromup) with fishmeal and with soybean at two temperatures and two inclusion levels. Inclusion levels of lupin and soybean were 15 and 25% at 14°C and 15% at 18°C. Diets were formulated to be isonitrogenous and isoenergetic on a gross compositional basis and to have a marginal crude protein content (40%). Inclusion of 15% reflected maximum industry inclusion rates for lupin where as 25% inclusion reflected a higher level in order to investigate whether performance changed at the higher level. The lower temperature of 14°C reflected an optimum summer temperature and was compared with an elevated summer temperature of 18°C, but one at which salmon would still be fed commercially in Tasmania (Carter et al., 2003; Miller et al., 2006).

23.2 Materials and Methods

23.2.1 Diets

Five diets were formulated to be isonitrogenous and isoenergetic on an "as is" basis, gross energy was approximately 20 MJ/kg and protein was marginally limiting at approximately 400 g/kg crude protein (Table 1). Lupin kernel meal (*L. angustifolius* cv Coromup) added at 15 (LM15) and 25% (KM25) and dehulled soybean meal (solvent-extracted US-origin soybean meal, WESFEEDS,Bentley, WA, Australia) added at 15 (SB15) and 25% (SB25) were compared with a diet containing mainly fish meal (Chilean anchovy meal, Skretting Australia, Cambridge, TAS, Australia) added at 55.5% (FM55). The dry ingredients were milled to 600 µm, mixed, extruded using a Wenger X185, and coated in oil (Refstie *et al.*, 2006).

23.2.2 Fish and experiment

Atlantic salmon post-smolts were divided between 24 300-l conical bottom tanks (10 per tank, 199.2 ± 7.8 g). The system was the same as described previously (Carter and Hauler 2000) except that it was divided into two, each system had temperature control and filtration systems. There were 15 tanks for the 14°C and 9 tanks for the 18°C treatments, respectively. At 14°C all diets were used (FM55, LM15, LM25, SB15 and SB25), at 18°C only the lower inclusion levels were used (FM55, LM15, SB15).

Fish were hand fed to appetite up to a set ration twice per day at 0900 and 1600, and daily feed intake recorded. Over the first 4 weeks feed intake was adjusted to ensure equal feed intake across the tanks. Fish were bulk weighed every 4 weeks for 12 weeks and then at week 15 when the growth experiment ended. At the end of the experiment fish were killed (overdose of benzocaine), wet weight and fork length measured and used for samples. Three fish per tank were used for whole body chemical composition (see below) and three fish per tank were dissected and used for gut histology (see below).

23.2.3 Apparent digestibility (AD)

Faecal samples were taken during week 16 by stripping (Austreng, 1978; Percival *et al.*, 2001) the fish remaining after some fish had been removed and used for all other samples, this ensured

fish used for gut histology had not been used for stripping since stripping may have affected the gut structure. As a precaution against stripping insufficient faecal material apparent digestibility was measured using settlement (Carter and Hauler, 2000) during week 11 to 12 and before the end of the growth experiment. Ytterbium oxide was included as an inert digestibility marker to calculate the digestibility of the diets for both AD experiments. The apparent digestibility (AD) values for protein was calculated using the standard formula: AD (%) = 100 – [100(%I_{diet} / %I_{faeces}) X (%N_{faeces} / %N_{diet})] (Maynard & Loosli, 1969), where I is the inert marker (Yb_2O_3) and N the nutrient.

23.2.4 Intestinal morphology

Three fish were randomly selected from each tank and were individually euthanased by benzocaine overdose. The liver and digestive tract was removed and stomach (with oesophagus attached), pylorus (fat removed), mid intestine and distal intestine were dissected and weighed at the end of the experiment to calculate organosomatic indices. A 2 cm section from the anterior distal intestine, just posterior to the ileorectal valve was sectioned, opened longitudinally and rinsed gently with 10% phosphate buffered saline, before fixing in 4% phosphate buffered formalin (Confix blue). The fixative solution was changed once after 24 h. All tissues were dehydrated prior to embedding in paraffin wax, and were sectioned at 5 µm. Tissue sections were stained with haemotoxylin and eosin and structure examined under light microscopy. Nine fish were sampled from each treatment, however initially for this report, results are based on one fish per tank (n=3). These data will be compared to gut function in relation to digestibility.

The morphology of the anterior distal intestine sections were assessed according to the following criteria, which have been used to classify conditions of SBM-induced enteritis in Atlantic salmon (Baeverfjord and Krogdahl, 1996): (1) widening and shortening of the intestinal folds, (2) loss of the supranuclear vacuolisation in the absorptive cells (enterocytes), (3) widening of the central lamina propria within the intestinal folds, with increased amounts of connective tissue and (4) infiltration of a mixed leucocyte population in the lamina propria and submucosa.

23.2.5 Chemical composition

Faecal samples were freeze dried pooled into one sample per tank and one sample per tank analysed for each of ytterbium (ICP-MS, Yb total by method iMET1STICP), crude protein (from the determination of total Nitrogen by SFA, based on %N x 6.25), crude fat (hexane extraction), phosphorous (ICP-AES) and ash. All analysis was conducted by the Chemistry Centre (WA), Department of Industry and Resources, Perth, Western Australia. Samples for chemical analysis were freeze dried. For faeces they were pooled into one sample per tank and analysed for ytterbium using a magnetic sector ICP-MS (Finnigan ELEMENT, Bremen, Germany). The instrument was operated in low resolution mode with ^{172}Yb isotope monitored. Prior to analysis the digested sample was further diluted (typically x10) with ultra-pure water with Indium added (100 ppb) as an internal standard. The method of external calibration was used for quantitation and calibration accuracy was confirmed via the analysis of an external quality control solution (AccuTrace Reference Standard, ICPM0165-5, New Haven, USA). Samples were analysed for: crude protein of faeces and diets (as total nitrogen, Thermo Finnigan EA 1112 Series Flash Elemental Analyser); crude protein of carcasses (Kjeldahl method with a copper catalyst, %N x 6.25); crude fat of diets and carcasses (Soxhtec - petroleum ether extraction); energy of diets and carcasses by bomb calorimeter (Gallencamp Autobomb, calibrated with benzoic acid); ash.

23.2.6 Statistical analysis

Means ± standard error (SE) are given and difference at probabilities of $P < 0.05$ assumed statistically significant. Since comparison of all treatments against all others treatments was of interest and the design was an incomplete block one-way ANOVA followed by Tukey HSD multiple comparison was used to compare treatments (SPSS version 14).

23.3 Results

23.3.1 Growth

There were no differences in survival between the treatments but there were significant differences between diets and temperatures in final weight and change in weight (Table 2). At 18°C there were no differences in growth between the diets. However, there was an effect of temperature and salmon fed LM15 and LM25 at 14°C had significantly higher growth than those fed FM55 and SB15 at 18°C. LM15 at 18°C was not different to any other treatment regardless of diet or temperature. Feed intake increased over the first weeks of the experiment and performance was compared between week 4 and 12, over this period there were differences in overall tank feed intake ($F = 3.70$, $P = 0.014$), weight specific feed intake (Table 2), and the change in weight (F 3.06, $P = 0.032$). There was no difference in efficiency, as FER (Table 2), suggesting differences in feed intake were the main driver of the growth differences. In addition, there was no major difference in protein digestibility and diet did not effect apparent digestibility crude protein between 14°C and 18°C (Table 2). However, SB25 at 14°C had a lower AD crude protein than either FM55 or LM15 at 18°C. There were no differences in carcass chemical composition between the treatments (Table 3).

23.3.2 Gastrointestinal tract

On a relative basis ($g.kg^{-1}$), the weights of organs differed between treatments (Table 4). The stomach was significantly heavier for LM15 at 14°C and lightest for LM15 at 18°C, but otherwise there were no interpretable trends with diet or temperature. The relative digestive tract weight was larger in the LM15 at 14°C, and smaller in the 18°C fish fed FM55 and LM15 and SB15. The relative weights of the mid intestine and distal intestine were not significantly different between the diets at the two temperatures. The proportional size of the mid intestine relative to the whole digestive tract (MI DTI) was significantly larger in the 18°C fish fed SB15, which was smaller than the SB15, LM15 and FM55 fed fish at 14°C. The distal intestine did not change in proportional size relative to the rest of the digestive tract (Table 4). The liver size relative to the whole body weight was largest in the fish held at 14°C and fed LM15 and SB25. All fish held at 14°C had larger livers. The fish held at 18°C fed FM55 and SB15 had the smallest livers (Table 4). The total digestive tract weight of fish fed the test feeds and held at 14°C were not significantly different in size to the FM55 fed fish held at the same temperature. Similarly there were no differences between the FM55 and test feed fed fish at 18°C, however the digestive tracts were notably smaller in fish held at 18°C compared to 14°C.

23.3.3 Intestinal morphology

No gross abnormalities were observed upon the external or internal surfaces of the digestive tract. Salmon fed FM55 at 14°C displayed no histopathological changes to mucosal structure (Fig 1A). Enterocytes were abundant in the mucosal epithelium. The lamina propria displayed no swelling and was of uniform width. Villi were uniform in shape and extended into the

intestinal lumen. Contrastingly, salmon fed FM55 at 18°C displayed a moderate reduction in the vacuolization of the intestinal mucosa (Fig 1B). Similarly, salmon fed LM15 and LM25 at 14°C displayed moderate vacuolization of the intestinal mucosa (Table 5). However, fish fed LM15 at 18°C showed severe changes to the intestinal mucosa with comparably less vacuolization. The intestinal folds appeared shorter than LM fed fish at 14°C and moderate widening of the lamina propria was observed, possibly due to a leucocytic infiltration. Fish fed SB15 at 14°C displayed a moderate reduction in vacuolization of the intestinal mucosa. All fish fed SB25 at 14°C and SB15 at 18°C showed moderate to severe intestinal morphological changes including reduced enterocyte abundance, widened lamina propria and shorter intestinal mucosal folds (Table 5).

23.4 Discussion

23.4.1 Growth performance

It was clear that both lupin diets out performed the fish meal and soy bean diets. Greater performance was largely driven by increased feed intake, although the experiment was designed to restrict differences between diets. This was broadly achieved on a tank basis and feeding what was assumed to be the correct ration. However equal feed intake is not always achieved, as was the case here, when feed intake is calculated retrospectively on a weight specific basis. Increased appetite and feed intake in response to lupin inclusion has been observed previously in Atlantic salmon (Carter & Hauler, 2000). It is likely that increased feed intake and the lower bulk density of lupin diets contributed to the tendency for increased stomach and gastrointestinal weights observed in the present research and previously (Glencross et al., 2004).

To investigate the data in more detail and because a replicate from the LM25 treatment had been lost which lead to some loss of resolution in the statistical analysis, a two-way ANOVA (diet and temperature) was used to investigate whether there were differences in growth performance between the fish meal and only the 15% inclusion diets (LM15 and SB15). There was no interaction between temperature and diet for any parameter (as in Table 2) analysed in this way. The key results were that for change in weight both diet ($P = 0.009$) and temperature ($P = 0.001$) were significant factors, LM15 and 14°C showed significantly higher change in weight. Weight specific feed intake was also significantly higher for LM15 but higher at 18°C. This meant growth efficiency was not different between diets but was lower at 18°C. Thus, in terms of growth performance LM15 appeared to be the better diet at both temperatures.

The relative size of organs in the gastrointestinal tract was affected by diet and temperature. Fish fed LM15 at 14°C generally had heavier digestive organs on a per body weight than fish held at 18°C fed FM55, LM15. Reftsie et al. (2006) also reported that salmon fed lupin kernel meal had higher intestinal tract weights (without stomach and pylorus), than fish fed fish meal and soybean fed fish (Refstie et al., 2006). Glencross et al. (2004) also reported an increase in rainbow trout gastrointestinal tract weight with increasing lupin concentration. Atlantic salmon fed various cultivars of lupin kernel meals, lupin protein concentrates and soybean meal produced no differences in MI weight and only slight differences in DI weight, where soybean had the lightest relative weight (Refstie et al., 2006). In contrast, the present study showed no differences in intestinal weights relative to the body weight. The MI relative to the remainder of the digestive tract was larger in SB15 fed fish at 18°C compared to FM55 and LM15 at 14°C. Further investigation of the histology of the gastrointestinal tract tissues is warranted to identify potential cause(s) for differential organ sizes.

23.4.2 Intestinal morphology

The morphology of the distal intestinal mucosa differed with dietary treatment consistent with previous descriptions of soybean meal induced enteritis (Baeverfjord and Krogdahl, 1996). Salmon fed FM55 at 14°C showed no histological abnormalities. Similarly, salmon fed fish meal at 12°C for 8 months showed no intestinal morphological changes (Sanden, 2005). However, fish held at 18°C and fed fish meal in the present study displayed moderate reduction in vacuolization of the mucosa. While no significant differences in intestinal histology were noted in *O. mykiss* fed lupin and fishmeal for 6 weeks at 17°C (Glencross et al., 2004), mild changes to intestinal structure were observed in Atlantic salmon fed fish meal (Refstie *et al.*, 2006). However, as in the present study, the intestinal mucosa of the salmon fed fish meal were the least affected by dietary treatment.

The inclusion of plant meals in salmon feeds and increasing temperature were associated with increased occurrence of pathological changes to the distal intestine mucosa. Soybean meal in feeds have previously produced soybean enteritis, and tissue changes in the present study were consistent with previous descriptions of this condition. The higher level of soybean meal inclusion particularly appeared to produce more pronounced intestinal tissue pathology, which was also reported in Atlantic salmon (Krogdahl *et al.*, 2003). Interestingly at 18°C both soybean and lupin treatments displayed similar degrees of mucosal alteration, suggesting further investigation of the distal intestinal changes with feeding lupin at high temperature may be warranted.

23.4.3 Conclusion

The current study assessed Atlantic salmon performance including gastrointestinal tract morphology and histology after feeding different protein sources at elevated temperature for an extended period. It has highlighted potential links between lupin meal inclusion and intestinal mucosal changes consistent with soybean meal enteritis, particularly at high temperature. However the observed pathological changes were increased at higher temperatures for all feed treatments to varying degrees. Considering the superior growth performance of the lupin meal fed fish, further investigation into the digestive consequences of pathological changes to the intestinal mucosa and effects on digestive function are warranted over longer periods of time at raised temperature, indicative of the commercial production cycle.

23.5 References

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Table 23.1 Ingredient and chemical composition of experimental Atlantic salmon feeds containing fish meal (FM), and lupin (LM) or soybean (SB) at two inclusion levels (15 and 25%).

	FM55	LM15	LM25	SB15	SB25
Ingredient composition (g/kg)					
Fish meal	555	458	394	453	385
Wheat flour	115	115	115	115	115
Lupin	0	150	250	0	0
Soybean	0	0	0	150	250
Fish oil	167	170	171	176	182
Pre-gel starch	83	59	59	59	59
Vit & Min Premix	8	8	8	8	8
Cellulose	71	39	2	38	0
Ytterbium oxide	1	1	1	1	1
Chemical composition (g/kg DM)					
Dry matter (g/kg)	935.3	921.0	940.0	934.7	934.6
Crude protein	405.0	407.7	414.2	410.4	430.1
Crude lipid	200.7	198.8	200.7	201.6	201.3
Ytterbium oxide	0.92	0.95	1.15	0.93	0.94
Gross energy	21.31	21.96	21.61	21.32	21.42

Table 23.2 The performance of Atlantic salmon fed diets containing lupin and soybean at two inclusion levels and at two water temperatures for 15 weeks.

		14°C					18°C				
		FM55	LM15	LM25	SB15	SB25	FM55	LM15	SB15	F	P
Initial Weight	g	197.4	196.9	193.5	202.8	197.3	205.9	198.9	201.1	0.67	0.694
		2.3	4.5	5.9	4.2	4.7	6.6	4.0	4.9		
Final Weight	g	357.7^{ab}	450.3^{b}	442.5^{b}	354.0^{ab}	361.7^{ab}	324.7^{a}	382.0^{ab}	319.7^{a}	3.28	0.025
		34.3	10.1	7.5	21.1	52.6	12.6	7.2	15.2		
Change in weight	g	160.3^{ab}	253.4^{b}	249.0^{b}	151.2^{ab}	164.4^{ab}	118.7^{a}	183.1^{ab}	118.5^{a}	3.96	0.012
		34.3	6.4	17.7	17.9	47.9	19.3	10.7	13.0		
Survival	%	96.7	96.7	80.0	90.0	96.7	96.7	96.7	86.7	0.81	0.587
		3.3	3.3	10.0	5.7	3.3	3.3	3.3	13.3		
Feed[1] intake	mg feed/ g/d	7.95^{a}	11.00^{bcd}	11.81^{d}	8.80^{abc}	8.96^{abc}	8.96^{abc}	11.19^{cd}	8.57^{ab}	7.67	0.001
		0.66	0.32	1.43	0.27	0.31	0.43	0.29	0.37		
FER[1]	g/g	0.94	0.90	0.78	0.78	0.76	0.68	0.76	0.67	2.02	0.120
		0.05	0.03	0.06	0.09	0.07	0.10	0.00	0.06		
AD CP	(%)	79.14^{ab}	80.91^{ab}	77.59^{ab}	77.87^{ab}	73.32^{a}	81.54^{b}	81.51^{b}	73.90^{ab}	3.20	0.028
		2.35	0.60	1.51	0.93	1.22	0.63	0.55	3.83		

Means ± SE (n=3).Means that are not significantly different share a similar superscript ($P < 0.05$, Tukey HSD)

[1] calculated from week 4 to week 12.

Table 23.3 Chemical composition of Atlantic salmon fed diets containing lupin and soybean at two inclusion levels and at two water temperatures for 15 weeks.

		14°C					18°C				
		FM55	LM15	LM25	SB15	SB25	FM55	LM15	SB15	F	P
Dry material	%	29.52	31.07	30.27	27.84	29.22	29.16	29.90	28.69	0.99	0.474
		1.08	0.12	1.39	1.38	1.50	0.64	0.22	0.80		
Crude protein	% DM	53.49	52.39	51.88	54.19	51.65	54.66	53.47	53.31	1.14	0.390
		0.81	1.13	2.68	0.81	0.92	0.86	0.45	0.48		
Crude lipid	% DM	34.09	36.71	35.57	33.63	36.24	32.11	32.90	32.47	1.91	0.138
		1.18	1.55	3.01	1.41	1.19	0.86	0.28	0.90		
Ash	% DM	7.02	6.55	6.48	7.26	6.62	7.39	6.59	7.44	2.55	0.061
		0.39	0.13	0.03	0.13	0.17	0.46	0.09	0.15		
Gross energy	kJ DM g^{-1}	23.957	24.906	24.073	24.546	24.204	24.181	24.435	23.896	1.68	0.189
		0.508	0.118	0.190	0.173	0.354	0.132	0.094	0.182		

Means ± SE (n=3).Means that are not significantly different share a similar superscript ($P < 0.05$, Tukey HSD)

Table 23.4 Relative organ weights and organosomatic indices for Atlantic salmon fed diets containing lupin and soybean at two inclusion levels and fed at two water temperatures.

		14°C					18°C				
		FM55	LM15	LM25	SB15	SB25	FM55	LM15	SB15	F	P
Digestive tract	g/kg	18.3 ^{ab}	22.1 ^{a}	16.3 ^{abc}	18.4 ^{ab}	16.4 ^{ab}	11.3 ^{c}	13.6 ^{bc}	11.6 ^{c}	5.56	<0.05
		1.2	2.4	1.7	2.3	1.5	0.7	1.0	0.8		
Stomach ^{a}	g/kg	15.37 ^{abc}	21.90 ^{a}	10.87 ^{bc}	16.97 ^{ab}	14.19 ^{abc}	7.96 ^{bc}	7.17 ^{c}	8.07 ^{bc}	5.80	<0.05
		2.33	3.26	2.06	2.38	3.11	0.41	0.31	0.70		
Digestive tract	g/kg	45.18 ^{abc}	54.26 ^{a}	39.69 ^{abc}	51.07 ^{ab}	48.41 ^{abc}	35.55 ^{c}	35.38 ^{c}	37.69 ^{bc}	4.412	<0.05
		3.22	5.11	2.75	3.33	4.18	2.17	1.62	4.44		
Liver	g/kg	22.55 ^{ab}	24.41 ^{a}	18.51 ^{abcd}	21.06 ^{abc}	24.38 ^{a}	14.07 ^{d}	16.13 ^{bcd}	14.63 ^{cd}	6.92	<0.05
		2.20	2.00	1.13	1.19	1.54	1.33	0.91	2.10		
Mid Intestine	g/kg	2.92	3.25	2.66	3.08	3.42	2.87	2.60	3.24	1.60	ns
		0.17	0.27	0.11	0.21	0.20	0.24	0.13	0.43		
Distal intestine	g/kg	5.08	5.43	4.84	4.99	4.79	4.40	4.30	4.70	0.87	ns
		0.29	0.33	0.45	0.39	0.32	0.33	0.20	0.71		
DI DTI ^{b}		114.67	103.13	121.30	101.04	102.80	126.32	122.59	122.59	1.94	ns
		6.25	5.28	5.61	10.43	8.37	10.56	5.42	5.10		
MI DTI ^{c}		65.91 ^{bc}	61.96 ^{c}	69.30 ^{abc}	62.02 ^{c}	72.71 ^{abc}	79.97 ^{ab}	73.67 ^{abc}	85.32 ^{a}	4.07	<0.05
		3.95	5.59	4.85	5.30	3.95	2.27	2.32	2.14		

Means ± SE. Means that are not significantly different share a similar superscript (P<0.05, Tukey HSD)

[a] Wet weight of stomach including oesophagus

[b] DI DTI = mid intestine somatic index = wet weight of mid intestine /whole digestive tract*100

[c] MI DTI = distal intestine somatic index = wet weight of distal intestine / whole digestive tract *100

Table 23.5 Visual discolouration of liver and anterior distal intestine of Atlantic salmon fed diets containing lupin and soybean at two inclusion levels and fed at two water temperatures.

		14°C					18°C		
		FM55	LM15	LM25	SB15	SB25	FM55	LM15	SB15
[1]Liver Discolour	%	44.4	66.7	11.1	33.3	0	33.3	0	0
[2]Anterior distal intestine	%	11.1	55.6	44.4	11.1	0	33.3	11.1	0

Means ± SE. Means that are not significantly different share a similar superscript ($P < 0.05$, Tukey HSD)

[1] liver discolouration = pale or mottled in colouration

[2] anterior distal intestine = darkened and visual swelling

Table 23.6 Morphological change to the anterior distal intestine structure of Atlantic salmon fed diets containing lupin and soybean at two inclusion levels and fed at two water temperatures.

	14°C					18°C		
	FM55	LM15	LM25	SB15	SB25	FM55	LM15	SB15
low	3	2	2	1	–	1	–	–
medium	–	–	1	2	1	2	1	1
severe	–	1	–	–	2	–	2	2
Total no	3	3	3	3	3	3	3	3

The changes were classified according to criteria defined by Baeverfjord and Krogdahl, (1996) as described in text.

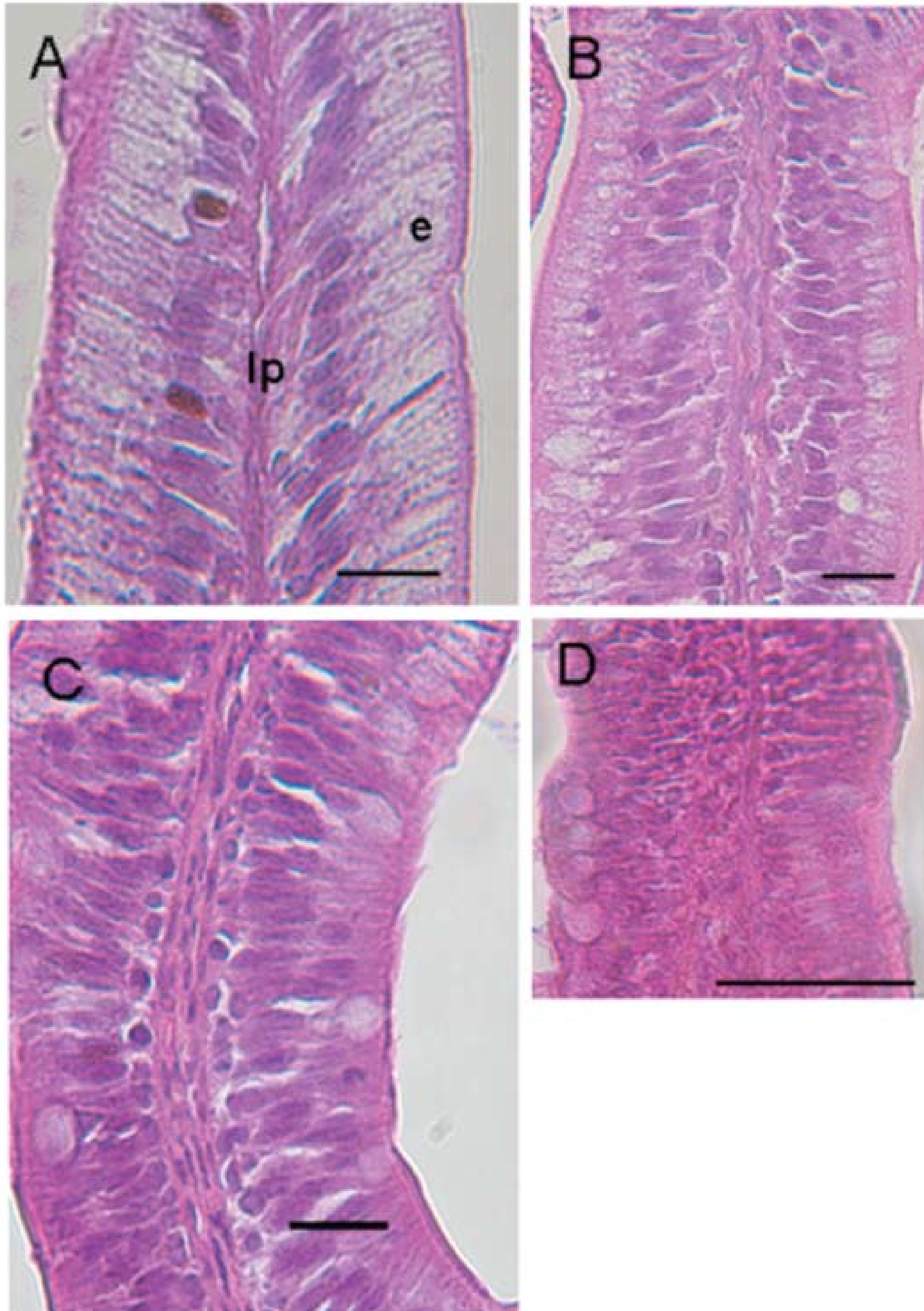

Figure 23.1 Histological detail of the anterior distal intestinal villous folds of Atlantic salmon fed (A) fish meal (FM55) at 14°C, (B) 25% lupin meal (LM25) at 14°C and (C) FM55 at 18°C and (D) LM15 at 14°C. Note the lamina propria (lp) of the villous folds, and (e) enterocytes (absorptive cells). The morphological changes in (A) are considered low, whereas (B) and (C) are considered moderate, and (D) severe (refer to table 6).

24.0 Digestive function and intestinal integrity in Atlantic salmon (*Salmo salar*) fed kernel meals and protein concentrates made from yellow or narrow-leafed lupins[a]

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Abstract

This study assessed the effects of yellow lupin (*Lupinus luteus*) and narrow-leafed lupin (*L. angustifolius*) kernel meals and protein concentrates on the gastrointestinal integrity, capacity for digestive hydrolysis, and digestibility of nutrients in Atlantic salmon. A basal diet (FM) was made from fish meal, wheat, and fish oil. Six additional diets were formulated by replacing 30% of the FM diet with lupin kernel meal made from *L. luteus* cv. Wodjil (LKM), *L. angustifolius* cv. Belara (BKM), and *L. angustifolius* cv. Myallie (MKM), lupin protein concentrates made from the same *L. luteus* (LPC) and *L. angustifolius* cv. M (MPC), or extracted soybean meal (SBM). All diets were extruded. Each diet was fed to three groups of 176 g salmon kept in 1 m^2 tanks with 5.6°C saltwater for three weeks prior to sampling of blood, intestinal organs, digesta, and faeces. Inclusion of lupin meals in the diets resulted in harder and more condensed feed particles. Ulcer-like lesions were observed in the stomach of fish from all feeding groups, and this was worsened by lupin in the diet, but did not appear to be related pellet hardness. No consistent altered morphology was observed in distal intestine (DI) of fish fed the FM and lupin diets, while the DI of fish fed SBM showed consistent and typical soybean meal-induced pathomorphological changes. Plasma cholesterol was higher when feeding MKM and LKM than when feeding FM, MPC, and LPC, with intermediate levels when feeding BKM and SBM. Feeding LKM and LPC resulted in a higher weight of the GIT when related to body weight. Trypsin activity and bile acid concentration were generally higher in digesta from the pyloric (PI) and mid (MI) intestine when feeding FM and lupin diets than when feeding SBM, while the opposite was seen for trypsin activity in digesta from DI. There were no effects of diet on leucine aminopeptidase (LAP) and maltase activity in PI and MI, but in DI the activity of these brush border enzymes were significantly lowered when feeding SBM. SBM in the diet resulted in watery faeces and lowered apparent digestibility of lipid, but this was not observed when feeding the lupin diets. To conclude, the tested lupin kernel meals and protein concentrates did not alter the intestinal function in Atlantic salmon when included at 30 % of the diet. Dietary lupin were, however, involved in the worsening of ulcer-like gastric lesions.

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24.1 Introduction

There is an ongoing effort to reduce the reliance on fish meal in aquaculture diets. Lupin (*Lupinus* spp.) meals are among the ingredients that provide potential for fish meal replacement by vegetable protein in fish feeds. Three lupin species are commercially produced and used as feed ingredients. These are the European white lupin (*L. albus*), the Australian narrow-leafed lupin (*L. angustifolius*), and the yellow lupin (*L. luteus*; Petterson, 2000). It is the dehulled kernel meals of the lupins that are mostly used in fish diets, and kernel meals of all three lupin species are reported to be of high nutritional value to salmonid fishes (De la Higuera et al., 1988; Gomes et al., 1995; Burel et al., 1998, 2000; Carter and Hauler, 2000; Farhangi and Carter, 2001; Glencross and Hawkins, 2004; Glencross et al., 2003a, 2003b, 2004a, 2006). Prototype lupin protein concentrates are also developed and have been tested in salmonids with promising results (Glencross et al., 2004b, 2005, 2006).

Effects of dietary lupin meals on digestive physiology and intestinal integrity in fish are, however, little investigated. Farhangi and Carter (2001) found an insignificant tendency for shortened villous height in the proximal intestine of rainbow trout (*Oncorhynchus mykiss*) with increasing inclusion of narrow-leafed lupin kernel meal in the diet, but did not histologically assess other intestinal sections. Apart for slightly higher relative gastrointestinal weight, Glencross et al. (2004a) also found no effects of dietary yellow lupin kernel meal on the histology of the intestine in rainbow trout. These fish were, however, preserved intact in formalin until dissection. As the intestinal wall requires rapid dissection and preservation after the fish is euthanised to avoid autolysis of the mucosa, the intestines of these fish may have been compromised before they were histologically assessed.

Soybean meals are extensively used in fish feeds, and effects of soy on digestive function and intestinal integrity in salmonids have been investigated in detail. It has been shown that soy contains a still unidentified heat stabile and alcohol soluble soy component(s) that cause pathomorphological changes in the distal intestine of salmonid fishes (van den Ingh and Krogdahl, 1990; van den Ingh et al., 1991, 1996; Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996; Burrells et al., 1999). This condition alters the digestive process by reducing the activity of membrane bound and cytosolic digestive enzymes in the mucosa (Krogdahl et al., 1995; Bakke-McKellep et al., 2000; Krogdahl et al., 2003), by reducing the carrier mediated nutrient transport (Nordrum et al., 2000), and by decreasing the absorption of macromolecules by the distal intestine (Bakke-McKellep, 1999). The latter apparently reduces the reabsorption of endogenous digestive secretions, as indicated by dramatically increased activity of trypsin in the distal intestinal contents (Dabrowski et al., 1989; Krogdahl et al., 2003).

Concomitant with this, but potentially unlinked, lowered faecal dry matter content and reduced digestibility of lipid is observed when feeding soybean meal to fish (Refstie et al., 1999, 2000, 2001, 2005, 2006). Soy contains components that bind bile acids in the intestine (Storebakken et al., 2000; Bakke-McKellep and Refstie, 2006), thereby potentially increasing the faecal steroid and lipid loss. In fish fed soybean meal this is indicated by lowered plasma cholesterol (Kaushik et al., 1995; Refstie et al., 1999), changes in cholesterol metabolising hepatic enzymes (Martin et al., 2003), and increased cholesterol requirement (Twibell and Wilson, 2004).

Any new feed ingredient for fish should, thus, be thoroughly tested with regard to digestive function alterations in relevant species before they are introduced in commercial diets. Based on this, the objectives of this work were to evaluate how dietary inclusion of different lupin kernel meals and protein concentrates made from the meals affected 1) the integrity of the

intestinal mucosa, 2) the capacity for nutrient hydrolysis, and 3) the apparent digestibility of nutrients in Atlantic salmon.

24.2 Material and methods

24.2.1 Ingredients and diets

The fish meal and the extracted and toasted soybean meal were supplied from Skretting Australia (Cambridge, TA, Australia). Kernel meal from *Lupinus luteus* cv. Wodjil was supplied from Coorow Seed Cleaners (Coorow, WA, Australia) while kernel meals from *L. angustifolius* cv. Belara and *L. angustifolius* cv. Myallie were supplied by Department of Agriculture (South Perth, WA, Australia). Lupin protein concentrates were made from the *L. luteus* cv. Wodjil and *L. angustifolius* cv. Myallie kernel meals at Department of Agriculture (South Perth) as described by Glencross et al. (2006), based on the extraction processes reported by Lasztity et al. (2001).

A basal diet (FM) was formulated from fish meal, wheat, and fish oil. Six additional diets were then formulated by replacing dry fish meal and wheat mix in the basal diet with one of the following meals in each diet: Lupin kernel meal made from *L. luteus* cv. Wodjil (LKM), *L. angustifolius* cv. Belara (BKM), or *L. angustifolius* cv. Myallie (MKM), lupin protein concentrate made from *L. luteus* cv. Wodjil (LPC) or *L. angustifolius* cv. M (MPC), or extracted soybean meal (SBM). Each diet was formulated to contain 30 % of the test ingredient. The dry ingredients were milled to < 600 µm before mixing. The diets were extruded on a Wenger X185 experimental scale extruder, dried to about 6 % moisture, and coated with oil at the Australian Experimental Stockfeed Extrusion Centre (Roseworthy College S.A., Australia). Composition of the diets is given in Table 24.1.

Bulk density of each diet was estimated as the average weight of one litre of feed after three repeated measurements. Average pellet diameter and length was measured by a calliper measuring 10 random pellets from each diet. Existing quality was measured by sifting two repeated samples of 100 g of each diet through a series of three sieves with mesh-width of 2.8, 0.5, and 0.0 mm for 30 seconds with 1.5 mm amplitude. Existing quality was calculated as the percent-wise proportion of the diet that remained in the 2.8 mm sift. Pellet durability (wear resistance) was estimated by a Ligno tester (Lignotech LT110, Borregaard UK Ltd., UK). Prior to the test repeated samples of 120 g of each diet was sifted as described for the measurements of existing quality. 100 g of sifted diet was then run in the Ligno tester for 240 seconds before being sifted again. Pellet durability index was calculated as the percent-wise proportion of the tested diet that remained in the 2.8 mm sift. Pellet breaking force was measured on 10 random pellets from each diet by diametric compression in a Lloyd texture analyser (Model 1000R, Hampshire, UK), fitted with a 500 N load cell and a PC-operated remote control. The pellets were positioned diametrically (laying) between two rigid plates, and submitted to an imposed compression displacement at a rate of 10 mm $min.^{-1}$. Hence, the force (kPa) applied on the pellet was progressively increased, and the load at breakage was recorded. Technical quality of the diets is given in Table 24.3.

24.2.2 Fish, rearing conditions and sampling

The experiment was conducted in accordance with laws and regulations that control experiments and procedures in live animals in Norway, as overseen by the Norwegian Animal Research Authority. The experiment was done at AKVAFORSK (Sundndalsøra, Norway), where seawater

adapted Atlantic salmon (*Salmo salar*) were fed the experimental diets for a total of 22 days. Prior to the experiment the fish were fed commercial diets (Skretting AS, Stavanger, Norway). At the onset of the experiment, 21 groups of salmon (176 g, 118 fish/group) were randomly distributed from a holding tank to fibreglass tanks (1 x 1 x 0.6 m, water depth 40-50 cm) supplied with seawater, and the experimental diets were randomly allocated to three groups of fish each. The fish were then fed the experimental diets for 21 feeding days. The fish were fed continuously (24 hr d^{-1}) by electrically driven disc feeders, aiming for 15% overfeeding based on expected feed intake. The water temperature during the experimental period was stabilised at 5.6°C, and the O_2 saturation of the outlet water was above 80%.

At feeding day 21, 20 fish randomly selected from each tank were euthanised in water with a lethal concentration of tricaine methanesulfonate (MS 222, Argent Chemical Laboratories Inc., Redmont, WA, USA), weighed individually, and the gastrointestinal tracts (GITs) were dissected out. Six fish per tank were sampled for analysis of alkaline phosphatase (ALP) and maltase activity. These GITs were sectioned into stomach (ST); pyloric intestine (PI), defined as the intestine from the most proximal to the most distal pyloric caeca; mid intestine (MI), defined as the intestine between the most distal pyloric caeca and the appearance of transverse luminal folds and increase in intestinal diameter, and; distal intestine (DI), defined as the region characterised by the transverse luminal folds and increased intestinal diameter to the anus. Surrounding adipose and connective tissue was carefully removed, the sections cut open and emptied (with the exception of the pyloric caeca) before frozen in liquid nitrogen and stored at -80°C.

Blood and intact intestines were furthermore taken from 12 of the fish sampled per tank. Blood was collected from the caudal vein into vacutainers containing anticoagulant (heparin). Samples were kept on ice until centrifugation at 3000 rpm for 10 minutes. Plasma samples were aliquoted into three separate eppendorf tubes, frozen in liquid nitrogen and stored at -80°C until analysis. The intact intestines were sampled for estimation of trypsin activity and bile acid concentration, and were wrapped in aluminium foil, frozen in liquid nitrogen and stored at -40°C. After allowing the GITs to partially thaw they were carefully opened by cutting with a scalpel. When the intestinal wall could easily be pulled away, the intestinal contents were removed and pooled per tank by intestinal section as described above for analysis. For this sampling the PI was further subdivided into proximal, PI1, and distal, PI2, portions, and the DI into proximal, DI1, and distal, DI2, portions.

From the last two fish sampled per tank, a 5 mm tissue sample was cut (a transverse cut relative to the length of the tract) from the central area of DI. These samples were placed and stored in phosphate-buffered formalin (4%, pH 7.2) for histological examination.

Faeces were stripped from the remaining fish in each tank as described by Austreng (1978). The faecal samples were pooled per tank and immediately frozen and stored at -20°C.

24.2.3 Chemical analyses

Plasma was analysed for glucose, alanine aminotransferase (ALT) activity, total protein, glucose, cholesterol, triacylglycerides, free fatty acids, inorganic phosphorus, calcium, sodium, and potassium according to standard methodology by the Central Laboratory at The Norwegian School of Veterinary Science. Faeces were freeze-dried (Hetosicc Freeze drier CD 13-2 HETO, Birkerød, Denmark) prior to analyses. Diets and freeze dried faeces were analysed for dry matter (105°C to constant weight), ash (combusted at 550°C to constant weight), nitrogen (Kjeltec Auto Analyser, Tecator, Höganäs, Sweden), amino acids (Biochrom 30 Amino Acid Analyser, Biochrom, Cambridge, UK, after hydrolysis according to EC Commission Directive

98/64/EC (1999)), lipid (pre-extraction with diethylether and hydrolysis with 4 M HCl prior to diethylether extraction (Stoldt, 1952) in a Soxtec (Tecator) hydrolysing (HT-6) and extraction (1047) apparatus), gross energy (Parr 1271 Bomb calorimeter, Parr, Moline, IL, USA), and yttrium (inductivity coupled plasma (ICP) mass-spectroscopy, as previously described by Refstie et al. (1997)). Diets were also analysed for starch (determined as glucose after hydrolysis by α-amylase and amylo-glucosidase, followed by glucose determination by the "GODPOD method" (Megazyme, Bray, Ireland)).

24.2.4 Enzyme and bile acid assays

Trypsin activity was determined colorometrically in freeze dried intestinal contents from PI, PI2, MI, DI1 and DI2. Trypsin activity was determined colorimetrically as described by Kakade et al. (1973) using the substrate benzoyl-arginine-p-nitroanilide (BAPNA; Sigma no. B-4875, Sigma Chemical Co., St. Louis, MO, USA) and a curve generated from a standardised bovine trypsin solution. Trypsin activity is expressed both as U mg^{-1} dry intestinal contents.

Bile acid concentration was also measured colorometrically in freeze dried contents from PI, PI2, MI, DI1 and DI2. A sample of 0.05 g from each intestinal area and fish was weighed out and diluted 1:40 with distilled water. The samples were mixed and incubated for 10 min on ice. A one ml sample was then sonicated for one min before centrifugation at 13,000 rpm for 10 min. The supernatant was drawn and enzymatic, colorimetric determination of total 3 α-hydroxy bile acids was done with a kit (Enzabile®, Nycomed, Oslo, Norway) using 3α-hydroxysteroid dehydrogenase and diaphorase in the presence of NAD^+, H^+, and nitrobluetetrazolium, with the resulting formazan formation read at 540 nm. The bile acid concentration was determined using a curve generated from a standardized taurocholic acid solution.

Activities of brush-border membrane bound leucine aminopoptidase (LAP) and maltase were determined in homogenates of intestinal tissue from PI, MI, and DI. The tissues were thawed, weighed and homogenized (1:20) in ice-cold 2 mM Tris/50 mM mannitol, pH 7.1, containing phenyl-methyl-sulphonyl fluoride (Sigma no. P-7626) as serine protease inhibitor. Aliquots of homogenates were frozen in liquid N and stored at -80°C prior to analysis. The LAP and maltase activities were determined colorimetrically as previously described by Krogdahl et al. (2003). Incubations were performed at 37°C. Enzyme activities are expressed as mmol (LAP) or µmol maltase substrate hydrolysed h^{-1} and related to g tissue and whole tissue and kg BW of the fish.

24.2.5 Histological examination

When opening the intact gastrointestinal tract for sampling of intestinal contents, macroscopically visible lesions were observed in the stomachs of the fish. The lesions appeared as focal or multifocal pale circular depressions with circumscribing red discoloration. The central depressions varied in diameter (pinpoint to 4 mm) and were observed in all areas of the stomach. The number of lesions was recorded and based on the diameter of the central depressions the lesions were categorised as small, medium or large.

Tissues were taken from the middle part of the DI, fixed in 10 % phosphate-buffered formalin, dehydrated in ethanol, equilibrated in xylene, and embedded in paraffin according to standard histological techniques. Sections of approximately 5 µm were cut and stained with haematoxylin and eosin before examination under a light microscope. Intestinal morphology was evaluated according to the following criteria: (1) widening and shortening of the intestinal folds (2) loss of the supranuclear vacuolisation in the absorptive cells (enterocytes) in the intestinal epithelium; (3) widening of the central lamina propria within the intestinal folds, with increased amounts

of connective tissue and (4) infiltration of a mixed leukocyte population in the lamina propria and submucosa. These are the characteristics of the condition previously described as soybean meal-induced enteritis in Atlantic salmon (Baeverfjord and Krogdahl, 1996).

24.2.6 Calculations

Crude protein (CP) was calculated as N x 6.25. Amino acid protein was estimated after hydrolysing the protein for amino acid analysis as the sum of dehydrated amino acids (as when peptide-bound). Apparent digestibility was estimated by the indirect method, as described by Maynard and Loosli (1969), using Y_2O_3 as an inert marker (Austreng et al., 2000).

24.2.7 Statistical analyses

The results were analysed by the General Linear Model procedure in the SAS computer software (SAS, 1985). Mean results per tank were subjected to one-way analysis of variance (ANOVA) with Diet as the independent variable. Prior to analysis, the percent-wise ulcer frequency per tank was arcsine transformed, the number of ulcers per affected fish was ln transformed, and the fish were also grouped according to lupin or no lupin in the diets. The results from the ANOVA are presented with the square root of the mean square error ($\sqrt{MSE}$) indicating variation. Significant differences among treatments were indicated by Duncan's multiple range test. The level of significance was chosen at $p \leq 0.05$, and the results are presented as group means (n=3).

24.3 Results

No fish died during the 21 days experimental feeding period. At feeding day 21 the mean weight of the fish randomly sampled from each feeding group ranged from 208 (fed the MKM diet) to 221 g (fed the FM diet). The mean filling of the sampled gastrointestinal tracts ranged from 23 (MPC diet) to 38 g (MKM diet), while the mean weight of the stripped faeces ranged from 33 (MPC diet) to 54 g (LKM diet) when pooled per tank.

24.3.1 Feed composition and technical quality

The higher concentration of protein in the lupin protein concentrates than in the lupin kernel meals and extracted soybean meal was clearly reflected by high protein content in the LPC, and MPC diet (Table 24.2). Lower protein concentrations in the narrow-leafed lupin kernel meals than in the yellow lupin kernel meal and the extracted soybean meal also gave slightly lower protein content in the MKM and BKM than in the FM, LKM and SBM diets. Due to higher addition of fish oil, the lipid content was higher in the FM diet than in the other diets. The lipid contents in the diets also reflected different contents in the ingredients, being lower in the SBM diet than in the lupin diets.

As shown in Table 24.2, the feed ingredients affected the technical quality of the diets. Addition of lupin kernel meals resulted in lower expansion following the high pressure moist extrusion and, thus, higher density of the diets. This was not seen when using lupin protein concentrates and extracted soybean meal in the diets. The lupin and soybean meals also resulted in longer pellets, potentially due to more rapid flow through the extruder. All diets contained little dust (0.01 to 0.41 g/kg, data not shown), but the diets containing lupin kernel meals were harder, as seen from high wear resistance (durability index) and force necessary to crush the pellets (breaking force). The diets containing lupin protein concentrates and extracted soybean meal were less wear resistant, but requiring high breaking force to be crushed.

24.3.2 Plasma chemistry

No differences in the activity of alanine aminotransferase (ALT) in the plasma, or in plasma concentration of triglycerides, free fatty acids, glucose, sodium, and potassium were observed among the feeding groups (Table 24.3). The plasma concentration of total protein was higher when feeding LPC and MPC than when feeding LKM and BKM, with intermediate concentrations when feeding the other diets. The plasma concentration of cholesterol was on the other hand lower when feeding LPC and MPC than when feeding LKM and BKM, with intermediate concentrations when feeding the other diets. The same pattern was seen for plasma concentrations of calcium. The plasma concentrations of inorganic phosphorus were lower when feeding LPC, MPC, and BKM than when feeding FM and MKM, with intermediate concentrations when feeding the other diets.

24.3.3 Gastrointestinal morphology

High frequencies of fish with macroscopically visible lesions in the stomachs resembling gastric ulcers were observed in all feeding groups (Table 24.4). Although the frequency of affected fish ranged from 17 % (fed FM) to 58 % (fed MPC), this difference was not statistically significant due to large variation among replicates within feeding groups. When, however, the feeding groups were grouped according to whether the diet contained lupin or not, the frequency of the gastric lesions was higher ($p<0.05$) in groups fed lupin diets (43.3 %) than in groups fed lupin free diets (19.4 %). In affected stomachs from 1 to 3 lesions of variable diameter were typically seen, and although numerically different among feeding groups, the variation among replicates within feeding groups was large also for this parameter. It was also similar if fish fed diets with or without lupin.

As judged by light microscopy, there were no consistent effects of the lupin kernel meals or protein concentrates on the morphology of the distal intestine (DI; Table 24.5). Most examined fish fed the FM and lupin diets showed normal morphology of the DI, characterised by the presence of well-differentiated enterocytes with many absorptive vacuoles. In contrast, all but one fish fed SBM showed severe morphological changes in the DI consistent with the soybean meal induced enteritis described by Baeverfjord and Krogdahl (1996). These changes included reduced vacuolisation of the enterocytes, reduced cell differentiation, variable degrees of inflammatory cell infiltration in the lamina propria/submucosa and shortening of the intestinal folds.

24.3.4 Relative organ weights

A larger pyloric intestine (PI) was found in fish fed LKM and LPC than in fish fed MPC and SBM, while the PI was of intermediate size in fish fed the other diets (Table 24.6). No effect of diet was observed on size of the mid intestine (MI). The DI was, however, larger in fish fed LKM, MKM, and BKM than in fish fed SBM, while it was of intermediate size in fish fed the other diets. Thus, the total intestinal tract was clearly larger in fish fed LKM than in fish fed FM, MPC, and SBM, and it was smaller in fish fed SBM than in fish fed all other diets except MPC.

24.3.5 Trypsin and bile acids in the digesta

The trypsin activity in the digesta differed among feeding groups along all sections of the intestinal tract (Table 24.7). In the first (PI1) and second (PI2) halves of the PI and in the MI the trypsin activity was generally lower in fish fed SBM than in fish fed the other diets, while the differences among fish fed the FM and lupin diets were small. In the first half (DI1) of the

DI the activity was higher in fish fed LPC than in fish from all other groups. In the second half (DI2) of the DI, however, the trypsin activity was higher in fish fed SBM than in fish fed all other diets. Among fish fed the FM and lupin diets, the trypsin activity was higher in fish fed LPC than in fish fed LKM, MKM, and BKM, and intermediate in fish fed FM and MPC.

The differences in bile acid concentration in the digesta were less distinct among feeding groups (Table 24.7). However, from PI1 to MI it was generally high when feeding BKM, LPC, and MPC, low when feeding SBM, and intermediate when feeding FM, LKM, and MKM. This was also seen in DI1, except that the bile acid concentration in fish fed BKM was relatively lower. In DI2 the bile acid concentration was low when feeding LKM, MKM, BKM, and SBM, high when feeding LPC, and intermediate when feeding FM and MPC.

24.3.6 Brush border enzymes

In PI and MI no differences were observed in activity of the brush border membrane-bound enzymes leucine aminopeptidase (LAP) and maltase (Table 24.8). In the DI, however, both the relative (measured g^{-1} tissue) and the total (measured in whole tissue kg^{-1} BW) LAP and maltase activity was significantly lower in fish SBM than in fish fed all other diets. Among fish fed the FM and lupin diets, both relative and total LAP and maltase activity was highest when feeding LKM, lowest when feeding MPC, and intermediate when feeding the other diets.

24.3.7 Apparent nutrient and energy digestibility

The dry matter content (DM) was highest in faeces of fish fed FM, lowest in fish fed SBM, and intermediate in fish fed the lupin diets (Table 24.9). Among groups fed the lupin diets the faecal DM content was highest when feeding LKM and MKM, lowest when feeding BKM, and intermediate when feeding and LPC and MPC.

The apparent digestibility of nitrogen was higher when feeding LPC and MPC than when feeding the other diets. The apparent digestibility of amino acid protein was also higher when feeding LPC and MPC than when feeding the other diets, but it was also higher when feeding FM and BKM than when feeding SBM, with intermediate estimates when feeding LKM and MKM.

The apparent digestibility of lipid was lower when feeding the FM and SBM diet than when feeding all lupin diets. The apparent digestibility of organic matter was highest when feeding the LPC and MPC diets, lower when feeding the FM diet, even lower when feeding the LKM and SBM diets, and lowest when feeding the MKM and BKM diets. The differences in apparent energy digestibility generally paralleled those of organic matter, although they were less distinct.

24.4 Discussion

The main findings in this experiment were that dietary kernel meals and protein concentrates made from yellow or narrow-leafed lupins, unlike extracted soybean meal, did not induce pathomorphological changes in the distal intestine, lower the trypsin activity and bile acid concentration in the pyloric and mid intestinal digesta, or reduce the digestibility of dietary lipid in Atlantic salmon when contributing 30 % of the diet. Dietary lupin did, however, worsen apparent gastritis in fish suffering from this. There was little effect of lupin species, cultivar, or product type on digestive function and intestinal integrity, but processing of lupin kernel meals into protein concentrates appeared to increase the availability of the lupin protein.

Uneaten feed was not collected and registered on a daily basis, so accurate feed intake could

not be quantified. However, as the ration was similar in all groups, as there were only small and inconsistent differences in body weight and gastrointestinal filling of fish sampled from each feeding group when terminating the experiment, and as similar quantities of faeces were obtained by stripping from all groups, the feed intake can be assumed to have been little affected by diet.

The lupin kernel meals were not thermally treated, as opposed to the fish meal and the toasted (steam dried) extracted soybean meal. Thus, the functional properties of the protein and/or polysaccharides in these meals were still intact, and the binding properties of the lupin meals resulted in condensed and hard pellets following extrusion. The manufacturing processes when making the protein concentrates denaturated the lupin proteins, and as the concentrates also contained very little carbohydrate, this resulted in less dense and durable pellets when extruding the lupin protein concentrate diets.

The differences in plasma protein and calcium concentrations among diet groups were small, and were not considered to be of biological significance. Plasma cholesterol was also little affected by diet, although slightly lowered when feeding the lupin protein concentrate diets. Similar plasma concentration of triglycerides, free fatty acids, and glucose indicated similar nutritional status in all feeding groups, and similar plasma sodium showed that all fish osmoregulated well. However, lupins contain little total and, thus, phytic acid bound phosphorus (Burel et al., 2000; Hertrampf and Piedad-Pascual, 2000), and the process when making lupin protein concentrates may have reduced this even more. As this was a short-term study, the phosphorus content in the diets was not adjusted by supplements. Plasma phosphorus in fish depends on phosphorus intake (Sugiura et al., 2004), and lower plasma concentration of inorganic phosphorus when feeding in particular lupin protein concentrate diets, thus, appear to reflect differences in dietary phosphorus.

As ulcer like lesions were observed in the stomach of fish from all feeding groups, this condition appears to have been caused by some constant dietary component(s). This may have been present in the vitamin and mineral premix. However, all diets also contained the same fish meal. Fish meals made from stale fish with high levels of the amino acid histidine contains high levels of histamine, which may react with lysine to form gizzerosine (Pike and Hardy, 1997). Both histamine and gizzerosine can induce gastric lesions in poultry and fish by stimulating hypersecretion of hydrochloric acid (Fairgrieve et al., 1994; Romero et al., 1994). Stale fish meal also contains some unidentified component(s) that cause pathological changes in the liver and intestine of Atlantic salmon (Opstvedt et al., 2000). Thus, poor fish meal quality appear the main explanation for the occurrence of gastric lesion. However, dietary lupin clearly worsened the condition, as the frequency of these lesions was twice as high when feeding lupin diets as when feeding the FM and SBM diets. There were no clear differences among groups fed lupin kernel meal or protein concentrates in this respect, indicating that mechanical stress by hard pellets was not the main ulcer inducing or worsening effect of dietary lupin. The effect of lupin on gastric morphology in salmonids should be further investigated.

The lack of histological changes in the distal intestine of salmon fed the lupin diets was paralleled by high activity of brush border leucine aminopeptidase (LAP) and maltase in this intestinal section as well as low luminal trypsin activity in the last half of the distal intestine. When feeding the SBM diet, typical soybean meal induced pathomorphological changes in the distal intestine (van den Ingh and Krogdahl, 1990; van den Ingh et al., 1991; Rumsey et al., 1994; Baeverfjord and Krogdahl, 1996) were followed by reduced LAP and maltase activity in the mucosa. This was in line with results from previous testing of soybean meal in salmonids (Krogdahl et al.,

1995; Bakke-McKellep et al., 2000; Krogdahl et al., 2003). Due to infiltration of inflammatory cells in the intestinal mucosa and the rapid regression of the condition following withdrawal of soybean meal from the diet, the condition have been classified as non-infectious, sub acute soybean meal-induced enteritis (Baeverfjord and Krogdahl, 1996), suggesting an etiology involving immunological mechanisms. An increased number of proliferating cells lining the villous folds of the distal intestine of soybean meal fed salmon (Sanden et al., 2005; Bakke-McKellep and Refstie, 2006) suggest disturbed functionality of enterocytes due to alterations in enterocyte turnover and degree of maturation. From the present study it appears that lupins do not contain component(s) inducing a similar condition in salmonids.

The tendency for increased relative weight (kg^{-1} BW) of the intestinal tract when feeding several lupin diets was in keeping with previous observations by Glencross et al. (2004a). It was then suggested that this was in response to lupin non-starch polysaccharides (NSP), supposedly shifting the balance between cell proliferation and cell death, apoptosis, and inducing gastrointestinal hypertrophy, as seen in pigs, rats and poultry (Simon, 2002). In the present study, however, a tendency for intestinal growth was also observed when feeding the diet with yellow lupin protein concentrate, which contained marginal NSP. Thus, this observation needs to be investigated in more detail and with a greater degree of experimental power. The reduced mass of the intestinal tract, and particularly of the distal intestine, when feeding the SBM diet was in keeping with Nordrum et al. (2000), and is a symptom of the soybean meal induced enteritis.

The trypsin activity in the digesta was more or less similar along the intestinal tract when feeding the FM and lupin diets. Thus, the secretion and reabsorption of pancreatic trypsin appeared little affected by dietary lupin or dietary protein content. When feeding the SBM diet, however, lower trypsin activity in the pyloric and mid intestine but significantly higher activity in the last part of the distal intestine indicated faecal trypsin losses and potentially exhaustion of the pancreatic capacity for trypsin synthesis. This was in keeping with Krogdahl et al. (2003), who found that the faecal trypsin increased in response to graded levels of soybean meal in the diet. It appears as a symptom of reduced functionality of the distal intestine and, thus, reduced capacity for reabsorption of digestive secretions in salmon fed soybean meal.

There were marginal differences in apparent protein digestibility when feeding the FM, SBM, and lupin kernel meal diets. The apparent protein digestibility was, however, higher when feeding the lupin protein concentrate diets despite higher protein content in the diets, similar trypsin activity in the digesta, and similar LAP activity in the mucosa. This indicates that processing the lupin kernel meals into protein concentrates increases the availability of the lupin protein to salmon.

Watery faeces and concomitant reduced lipid digestibility are typical effects of dietary soybean meal in Atlantic salmon (Refstie et al., 1999, 2000, 2001, 2005). This was thought to be mainly in response to indigestible and osmotically active soy α-galactoside sugars (Arnesen et al., 1990; Krogdahl et al., 1995) and/or indigestible and viscous soluble soy NSP (Refstie et al., 1999, 2005). However, lupins meals contain as much or in some cases more of similar α-galactoside sugars and NSP than that observed in extracted soybean meals (Bach-Knudsen, 1997; Kocher et al., 2000; Glencross et al., 2003a). Thus, as the faeces of the present fish was significantly drier when feeding lupin diets than when feeding SBM, and as the apparent lipid digestibility was high when feeding all lupin diets, the indigestible carbohydrates are apparently not the major components causing diarrhoea and lowered lipid absorption in Atlantic salmon fed soybean meals.

However, soybean meals also contain components like saponins and phytosterols that potentially bind bile acids in the intestine (Storebakken et al., 2000; Bakke-McKellep and

Refstie, 2006), thereby causing faecal steroid and lipid losses. In soybean meal-fed fish this is indicated by lowered plasma cholesterol (Kaushik et al., 1995; Refstie et al., 1999), changes in cholesterol metabolising hepatic enzymes (Martin et al., 2003), and increased cholesterol requirement (Twibell and Wilson, 2004). Despite low concentration of bile acids in the digesta and concomitant low apparent lipid digestibility when feeding the present SBM diet, plasma cholesterol and lipid concentration was little affected, as discussed above. Although bile acids appeared to be drained by faecal losses, a three-weeks feeding period, thus, appeared too short to induce noticeable effects of dietary soybean meal on plasma cholesterol.

The component(s) causing faecal drainage of bile acids were apparently not present or present at lower concentrations in the lupin meals. This was seen from a high bile acid concentration in the upper intestine but low concentration in the distal intestine when feeding the lupin kernel meal diets, indicating efficient enterohepatic recirculation of steroids. Plasma concentration of cholesterol and apparent lipid absorption was, thus, normal in these fish. The high concentration of bile acids along the whole length of the intestinal tract in fish fed the lupin protein concentrate diets may, however, indicate some faecal losses of bile acids in these fish, as it also corresponded with lowered plasma cholesterol despite high apparent lipid absorption. As these diets contained more lupin protein, it may indicate that some bile acid-binding component(s) were concentrated with the protein when making the lupin protein concentrates.

Although the intestinal bile acid concentration was generally lower when feeding the FM basal diet, a similar distribution of bile acids was seen along the intestinal tract as when feeding the lupin protein concentrate diets. In these fish plasma cholesterol was less affected, but the apparent lipid absorption was lowered. The FM diet contained about 25 % more lipid than the other diets, but as feeds for salmon of this size typically contains 30 % lipid it is unlikely that this was the main cause for the low lipid digestibility. However, as discussed in detail above, the gastric lesions observed in the present experiment indicated poor freshness of the dietary fish meal. If so, this may have affected the lipid digestibility negatively when feeding the FM diet, which contained as much as 70 % of the fish meal. When evaluating fish meal quality the focus is traditionally on protein digestion and amino acid absorption (Pike et al., 1990; Anderson et al., 1992, 1993, 1995, 1997). As the general understanding of digestive function and interactions in fish increases, more information of effects of fish meal quality on general digestive function and potential intestinal damage is needed.

The apparent digestibility of organic matter was lower when feeding the lupin kernel meal and SBM diets than when feeding the FM and lupin protein concentrate diets. Legume NSP is well known to be indigestible by Atlantic salmon (Refstie et al., 2005), so this reflected the NSP content in the crude vegetable meals and, thus, the diets based on these. A similar pattern was seen for apparent energy digestibility. The energy digestibility was, however, more affected by the differences in lipid digestibility, resulting in a relatively low energy digestibility when feeding the FM diet.

To conclude, use of lupin kernel meals in extruded diets resulted in condensed, harder, and more wear resistant feed particles. Gastric lesions resembling ulcers were observed in salmon from all feeding groups, but although stale fish meal was suspected, the causative agent for this remains unclear. The condition was also worsened by dietary lupin, and this was not related to feed pellet hardness. There was little effect of different lupin species, cultivars, or products on digestive function and intestinal integrity in the salmon. No lupin kernel meals or protein concentrates altered the morphology of the salmon intestine, but the SBM induced pathomorphological changes in the last half of the distal intestine. These SBM induced pathomorphological changes

also concurred with a lower activity of brush border membrane bound enzymes and apparently lowered reabsorption of pancreatic trypsin in this intestinal section. In the other intestinal sections trypsin activity was generally higher along the intestinal tract when feeding the FM and lupin diets than when feeding the SBM diet. Higher bile acid concentration in salmon fed the lupin diets than in salmon fed the FM or SBM diets was reflected in higher apparent digestibility of lipid. The protein in the lupin protein concentrates also appeared more available than the protein in the lupin kernel meals.

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Tables and Figures

Table 24.1 Composition of the diets.

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM
Formulation, g/kg							
Fish meal[a]	700.0	490.0	490.0	490.0	490.0	490.0	490.0
Wheat flour[a]	144.0	100.8	100.8	100.8	100.8	100.8	100.8
Fish oil[a]	150.0	105.0	105.0	105.0	105.0	105.0	105.0
L. l. cv. Wodjil kernel meal[b]		300.0					
L. a. cv. Myallie kernel meal[c]			300.0				
L. a. cv. Belara kernel meal[d]				300.0			
L. l. cv. Wodjil protein concentrate[e]					300.0		
L. a. cv. Myallie protein concentrate[f]						300.0	
Extracted soybean meal[a]							300.0
Vitamin and mineral premix[g]	5.0	3.5	3.5	3.5	3.5	3.5	3.5
Yttium oxide[h]	1.0	0.7	0.7	0.7	0.7	0.7	0.7
Chemical composition							
Dry matter (DM), g/kg	935.6	913.7	915.3	922.8	951.5	935.8	945.5
On dry basis (g/kg)							
Crude protein (CP), g	510.9	517.9	479.6	491.9	628.7	591.7	519.1
Amino acid protein[i], g	403.7	414.6	387.9	395.2	495.8	482.9	427.1
Lipid, g	248.9	205.8	201.0	204.3	205.4	218.9	174.2
Ash, g	124.9	97.5	94.2	98.5	94.1	92.1	108.6
Organic matter[j], g	875.1	902.5	905.8	901.5	905.9	907.9	891.4
Energy, MJ	23.4	22.9	22.9	22.8	23.5	23.7	22.3

[a] Skretting Australia (Cambridge, TAS, Australia)

[b] Yellow lupin (*Lupinus luteus* cv. Wodjil) kernel meal (Coorow Seed Cleaners, Coorow, WA, Australia)

[c] Narrow-leafed lupin (*L. angustifolius* cv.Myallie) kernel meal (Department of Agriculture, South Perth, WA, Australia)

[d] Narrow-leafed lupin (*L. angustifolius* cv. Belara) kernel meal (Department of Agriculture)

[e] Yellow (*L. luteus* cv. Wodjil) lupin protein concentrate made from the LKM (Department of Agriculture)

[f] Narrow-leafed (*L. angustifolius* cv. Myallie) lupin protein concentrate made from the MKM (Department of Agriculture)

[g] Rhone Poulenc (Goodna, QLD, Australia). In premix, kg^{-1}: retinol, 2.5 MIU; cholecalciferol, 0.25 MIU; α-tocopherol, 16.7 g; menadione, 1.7 g; thiamine, 2.5 g; riboflavin, 4.2 g; niacin, 25 g; pantothenic acid, 8.3;pyridoxine, 2.0 g; folic acid, 0.8; methylcobalamine, 0.005 g; biotin, 0.17 g; ascorbic acid, 75 g; choline, 166.7 g; inositol, 58.3 g; ethoxyquin, 20.8 g; copper, 2.5 g; ferrous iron, 10.0 g; magnesium, 16.6 g; manganese, 15.0 g; zinc, 25.0 g

[h] Stanford Materials Corporation (Aliso Viejo, CA, USA)

[i] Expressed as the sum of peptide-bound (dehydrated) amino acids

[j] Calculated by difference

Table 24.2. Technical quality of the diets.

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM
Bulk density, g l^{-1}	664	745	745	708	618	683	597
Pellet diameter, mm	5.9	5.3	5.3	5.5	5.6	5.5	5.6
Pellet length, mm	4.9	6.2	5.7	5.5	6.6	5.7	6.4
Existing quality[a], %	99.9	100.0	100.0	100.0	99.3	100.0	99.6
Pellet durability index[b], %	87.5	99.8	98.5	97.3	5.0	25.6	0.9
Breaking force[c], kPa	398	757	818	805	745	688	585

[a] Diet remaining in a 2.8 mm sift after sieving for 30 seconds

[b] Diet remaining in a 2.8 mm sift after Ligno testing and sieving for 30 seconds

[c] Measured by a Texture analyser

Table 24.3 Plasma chemistry (mean, n=3).

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM	$\sqrt{MSE}$
ALT*, U l^{-1}	25.1	17.6	20.7	18.8	20.1	20.9	20.9	4.1
Total protein, g l^{-1}	37.7^{ab}	40.1^{a}	39.4^{a}	38.2^{ab}	36.6^{b}	36.3^{b}	38.9^{ab}	1.5
Cholesterol, mM	7.0^{bc}	7.8^{ab}	7.8^{a}	7.5^{abc}	6.7^{c}	6.8^{c}	7.0^{bc}	0.4
Triglycerides, mM	2.0	2.3	2.2	2.1	2.0	2.0	1.8	0.2
Free fatty acids, mM	0.4	0.5	0.4	0.5	0.5	0.3	0.4	0.2
Glucose, mM	5.8	5.6	5.6	5.5	5.3	5.2	5.9	0.5
Na, mM	166.3	165.9	168.8	166.6	167.3	164.6	165.9	3.0
K, mM	3.4	2.9	2.0	3.1	3.6	3.4	3.6	1.0
Ca, mM	2.9^{ab}	2.9^{ab}	3.0^{a}	2.9^{ab}	2.8^{b}	2.8^{b}	2.9^{ab}	0.1
Inorganic P, mM	3.3^{a}	2.7^{bc}	3.2^{ab}	2.4^{c}	2.6^{c}	2.6^{c}	2.8^{abc}	0.3

$\sqrt{MSE}$ is the square root of the mean square error in the ANOVA

[abc]different superscripts indicates a statistical difference ($P \leq 0.05$)

* Alanine aminotransferase

Table 24.4 Frequency of gastric lesions resembling ulcers (mean, n=3).

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM	$\sqrt{MSE}$
Fish with lesions, %	16.7	41.7	58.3	27.8	38.9	50.0	22.2	0.2
Lesions per affected fish								
Small	0.72	0.48	0.29	0.00	0.46	0.10	0.42	0.30
Medium	0.89^{y}	0.79^{y}	1.67^{xy}	2.65^{x}	1.32^{xy}	2.27^{xy}	0.92^{xy}	0.40
Large	0.00	0.03	0.22	0.08	0.12	0.14	0.17	0.10
Total number	1.61	1.30	2.18	2.73	1.90	2.51	1.51	0.30

$\sqrt{MSE}$ is the square root of the mean square error in the ANOVA

[xy]different superscripts indicates a statistical tendency ($P \leq 0.1$)

Table 24.5 Histological scores for distal intestinal structure.

Diet	Number of screened fish	Normal structures	Moderate changes[1]	Severe changes[2]
FM	6	3	2	1
LKM	6	6		
MKM	6	6		
BKM	6	4	1	1
LPC	6	5		1
MPC	6	3	1	2
SBM	5	1		4

[1] Reduced vacuolisation of the epithelium

[2] Reduced vacuolisation of the enterocytes, reduced cell differentiation, variable degrees of inflammatory cell infiltration in the lamina propria, shortening of the villi, and sub-epithelial oedema

Table 24.6. Mean relative weights (g/kg BW) of different sections of the intestinal tract.

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM	$\sqrt{MSE}$
Pyloric intestine (PI)	17.2[abc]	18.3[a]	18.1[ab]	17.5[abc]	18.5[a]	16.7[bc]	16.5[c]	0.8
Mid intestine (MI)	2.2	2.0	2.0	2.1	2.2	2.1	2.0	0.1
Distal intestine (DI)	4.3[abc]	4.9[a]	4.4[ab]	4.8[ab]	4.2[bc]	4.2[bc]	3.7[c]	0.3
Total IT	23.7[bc]	25.3[a]	24.4[ab]	24.5[ab]	24.9[ab]	23.0[cd]	22.3[d]	0.8

$\sqrt{MSE}$ is the square root of the mean square error in the ANOVA

[abcd]different superscripts indicates a statistical difference ($P \leq 0.05$)

Table 24.7 Trypsin activity and bile acid concentration in dry contents from the first and second halves of the pyloric intestine (PI1 and PI2), the mid intestine (MI), the first and second halves of the distal intestine (DI1 and DI2), and the total intestinal tract (IT; mean, n=3).

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM	$\sqrt{MSE}$
Trypsin activity, U mg^{-1}								
PI1	188.9[a]	178.3[a]	210.0[a]	152.4[ab]	206.4[a]	216.4[a]	90.5[b]	44.2
PI2	143.0[b]	168.0[ab]	166.8[ab]	169.5[ab]	194.6[a]	173.6[ab]	99.8[c]	23.6
MI	156.7[a]	160.2[a]	128.4[ab]	145.9[a]	159.4[a]	156.4[a]	95.0[b]	23.9
DI1	68.9[b]	85.3[b]	66.5[b]	66.9[b]	112.8[a]	83.9[b]	69.6[b]	15.5
DI2	34.0[bc]	23.1[cd]	15.8[d]	18.0[cd]	49.3[b]	34.9[bc]	74.8[a]	9.5
Bile acid concentration,mg g^{-1}								
PI1	66.6[ab]	75.5[a]	61.5[ab]	98.0[a]	96.8[a]	103.8[a]	24.3[b]	23.7
PI2	44.2[cd]	63.4[abc]	54.6[bc]	81.0[a]	82.0[a]	75.2[ab]	25.8[d]	13.6
MI	49.6[bc]	63.6[ab]	51.5[bc]	77.1[ab]	87.3[a]	73.6[ab]	27.8[c]	14.9
DI1	36.5[abc]	33.4[bc]	25.4[bc]	32.4[bc]	53.6[a]	44.6[ab]	21.1[c]	10.2
DI2	20.6[bc]	14.7[cd]	8.5[d]	13.7[cd]	29.6[a]	24.1[ab]	12.7[cd]	4.8

$\sqrt{MSE}$ is the square root of the mean square error in the ANOVA

[abcd]different superscripts indicates a statistical difference ($P \leq 0.05$)

Table 24.8 Leucine aminopeptidase (LAP) and maltase activity in the pylorus intestine (PI), mid intestine (MI), distal intestine (DI) and the total intestinal tract (IT; mean, n=3).

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM	$\sqrt{MSE}$
Relative LAP activity, mmol h^{-1} g^{-1} tissue								
PI	9.8	10.4	10.0	11.1	8.8	8.0	8.8	1.6
MI	6.2	5.7	6.3	6.3	5.6	6.7	5.0	0.9
DI	8.6^{ab}	8.9^{a}	8.4^{ab}	8.0^{ab}	7.2^{ab}	6.4^{b}	2.5^{c}	1.2
Total LAP activity, mmol h^{-1} in whole tissue kg^{-1} BW								
PI	171.1	192.9	178.0	204.4	157.4	136.4	157.4	33.2
MI	13.4	11.8	13.2	12.4	12.0	14.2	10.7	2.1
DI	38.8^{ab}	43.9^{a}	39.3^{ab}	38.2^{ab}	32.9^{ab}	28.1^{b}	8.9^{c}	6.7
Relative maltase activity, μmol h^{-1} g^{-1} tissue								
PI	1.10	1.29	1.32	1.07	1.09	0.99	1.08	0.25
MI	0.69	0.71	0.71	0.69	0.71	0.71	0.65	0.07
DI	0.53^{ab}	0.71^{a}	0.67^{ab}	0.61^{ab}	0.52^{ab}	0.47^{b}	0.12^{c}	0.12
Total maltase activity, μmol h^{-1} in whole tissue kg^{-1} BW								
PI	18.7	23.9	23.7	18.7	20.0	16.7	18.8	5.1
MI	1.4	1.4	1.4	1.4	1.4	1.4	1.3	0.1
DI	2.3^{ab}	3.4^{a}	3.0^{ab}	2.0^{ab}	2.4^{ab}	2.0^{b}	0.4^{c}	0.6

$\sqrt{MSE}$ is the square root of the mean square error in the ANOVA

^{abc}different superscripts indicates a statistical difference ($P \leq 0.05$)

Table 24.9 Faecal dry matter (DM) and apparent digestibility of nutrients (mean, n=3).

Diet code	FM	LKM	MKM	BKM	LPC	MPC	SBM	$\sqrt{MSE}$
Faecal DM, %	15.5^{a}	14.4^{b}	14.3^{bc}	13.3^{d}	13.5^{cd}	13.7^{bcd}	10.7^{e}	0.5
Apparent digestibility, %								
Nitrogen	83.6^{b}	84.3^{b}	83.8^{b}	84.9^{b}	87.9^{a}	88.1^{a}	84.0^{b}	0.8
Amino acid protein	88.7^{b}	88.1^{bc}	88.1^{bc}	88.9^{b}	91.3^{a}	91.3^{a}	87.3^{c}	0.6
Lipid	77.2^{b}	85.9^{a}	86.1^{a}	87.7^{a}	82.9^{a}	86.3^{a}	76.5^{b}	3.0
Organic matter	74.1^{b}	70.4^{c}	67.4^{d}	68.2^{d}	78.6^{a}	79.6^{a}	69.0^{cd}	1.1
Energy	79.2^{b}	77.2^{bc}	76.1^{c}	76.6^{bc}	82.6^{a}	84.6^{a}	75.1^{c}	1.6

$\sqrt{MSE}$ is the square root of the mean square error in the ANOVA

^{abcd}different superscripts indicates a statistical difference ($P \leq 0.05$)

25.0 Digestibility of lupin kernel meals in feeds for the black tiger prawn, *Penaeus monodon*[a]

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Abstract

In recent years, new cultivars of lupins have largely replaced the cultivars that were studied in previous research with prawns. There was a need to establish if the breeding programs had introduced changes in the new lupin cultivars that would affect the nutritional value of the kernel meal for prawns. We have determined the apparent dry matter digestibility (ADMD), apparent crude protein digestibility (ACPD) and apparent digestibility of energy (ADE) of the yellow lupin *Lupinus luteus* cv. Wodjil, as well as of six of the new cultivars of *Lupinus angustifolius* when used in diets for the black tiger prawn, *Penaeus monodon*. The *L. angustifolius* cultivars represent about 80% of Australia's lupin production. We have also determined the apparent digestibility (AD) of the amino acids of five of the new cultivars of *L. angustifolius,* and of *L. luteus*, cv. Wodjil. Ytterbium acetate was used as an inert digestibility marker at a concentration of 0.5 g/kg in the diets. During the periods when faeces were collected, the prawns were fed every 6 h and faeces were collected within 3 h of being passed. Six replicate tanks were assigned to each treatment. The kernel meal from *L. luteus* cv. Wodjil had the highest ADMD (70.0%) and ADE (79.9%) but its ACPD was mid-range at 93.8%. The ADMD of the *L. angustifolius* kernel meals varied between 56.5% and 66.3% with the mean (± s.e.m.) of 62.6% (± 0.95%), and the ADE varied between 69.6% and 77.2% (mean ± s.e.m. = 74.0% ± 0.72%), whereas the ACPD varied between 92.7% and 96.8% (mean ± s.e.m.= 94.3% ± 0.48%). The AD of the amino acids was similar to the ACPD value. Though there were significant differences among the ADs of the new cultivars of *L. angustifolius,* their values are similar to, though slightly lower than the AD reported for the older cultivar, Gungurru. The general consistency of the *L. angustifolius* AD results suggests that nutritionists and feed formulators can confidently use mean AD values for dry matter, protein and energy for kernel meals comprising of random mixtures of cultivars.

25.1 Introduction

The availability of nutrients in the feed and its component ingredients is of prime interest to nutritionists and feed formulators. Though the gross chemical composition will give an indication of the nutrients present in a feed or in an ingredient, the digestibility of the nutrients gives a much better estimate of their availability. With any new ingredient, its nutrient composition and the digestibility of its key nutrients need to be determined before it can be used with best effect in nutritionally-based feed formulations.

Fishmeal has long been the main protein source used in feeds for most aquaculture species. However, with the increasing cost and periodic shortages of fishmeal on the global markets, the aquaculture industry is interested in reducing its dependence on fishmeal through the

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development of alternative protein sources (New and Wijkström, 2002). Lupins are a useful, protein-rich ingredient, which can partially replace fishmeal in feeds for both fish and prawns (Hughes, 1991; Burel et al., 1998; Smith et al., 2000). As Australia contributes about 80% of the global production of lupins, there has been a significant research effort in this country to evaluate lupin products in aquaculture feeds (Allan and Rowland, 1998; Smith, 1998; Carter and Hauler, 2000). Lupin kernel meal was found to be better digested than the whole seed meal, and its protein was found to be highly digestible (Smith, 1998; Booth et al., 2001; Glencross, 2001). Much of the early work was carried out using kernel meals derived from the narrow leafed lupin, *Lupinus angustifolius,* particularly a variety (or cultivar) called Gungurru. During the 1990's, Gungurru was the most widely-grown cultivar in Australia. Since then, lupin breeding programs have produced new cultivars that are better suited to the soil types and climatic conditions found in the different growing regions. Gungurru has been largely replaced by these new cultivars and now represents < 5% of Australian production (B. Buirchell, WA Agriculture. pers. comm.; Pulse Australia, 2006). In studies with rainbow trout, *Oncorhynchus mykiss*, Glencross and co-workers have determined the digestibility of the kernel meal from a number of the new cultivars of *L. angustifolius* and of the yellow lupin, *L. luteus* cv. Wodjil (Glencross et al., 2003; Glencross and Hawkins, 2004). These studies showed that the digestibility of protein in the kernel meals was generally high (85 to 90%). However, there have been no studies reported which have examined the response of any species of prawn to these new cultivars.

In this study with the black tiger prawn, *Penaeus monodon,* we have determined the apparent dry matter digestibility (ADMD), apparent crude protein digestibility (ACPD) and apparent digestibility of energy (ADE) of the yellow lupin *L. luteus* cv. Wodjil, and six of the new cultivars of *L. angustifolius* which represent about 80% of Australia's lupin production. We have also determined the apparent digestibility of the amino acids (excluding tryptophan) of *L. luteus*, cv. Wodjil and of five of the new cultivars of *L. angustifolius.*

25.2 Materials and Methods

25.2.1 Lupin kernel meals

Samples of whole-seed *L. angustifolius* lupins were obtained from the Department of Agriculture – Western Australia, lupin breeding program. The lupins were grown at either of two research field stations, Katanning (33.69 S, 117.61 E) and Wongan Hills (30.89 S, 116.72 E). Both batches of seed were obtained from the 2003 crop season. The seed was harvested and segregated by source and variety and stored at 4°C prior to processing. In addition, a sample of *L. angustifolius* cv. Myallie and a sample of *L. luteus* cv. Wodjil, both of which had been grown in the northern growing area near Coorow (29.88 S, 116.02 E) in the 2002 season, were obtained from Coorow Seed Cleaners (Corrow, WA). For processing the seed was graded according to seed size using round-holed 7mm, 6mm and 5mm sieves and each segregation, of each variety, separately split using a disc-mill dehulling unit (Department of Agriculture, South Perth, WA, Australia). The split (dehulled) segregation of each variety was then pooled prior to aspiration (air stream mediated density classification) to remove the hulls from the kernels. Any remaining seed hull fragments were manually removed to ensure a 100% pure preparation of seed kernels of each variety. The kernels were then rotor-milled (Retsch, Haan, Germany) through a 750 µm screen.

Two experiments were carried out to determine the digestibility of lupin kernel meals from a total of seven cultivars of *L. angustifolius* and one of *L. luteus*. The first experiment examined

the digestibility of *L. angustifolius* cultivars grown in the south of the Western Australian wheat belt, at Katanning. These cultivars were Kalya, Mandelup, Walan 2173, Myallie, Tanjil and the older cultivar Gungurru (Table 25.1). The second experiment examined kernel meals from lupins grown in the main (northern) growing areas of the wheat belt, at Wongan Hills. These were the *L. angustifolius* cultivars Kalya, Mandelup, Tanjil and Wonga. Also included were kernel meals from the cultivar Myallie and from *L. luteus* cv. Wodjil (Table 25.1 and 25.2).

25.2.2 Experimental animals

Juvenile prawns, *P. monodon*, were obtained from commercial prawn farms in northern Queensland, Australia. They were reared in the laboratory from about 2 g until they were > 12 g before being used in the digestibility experiments. During the grow-out period, the shrimp were maintained in 2500 L holding tanks with flow-through seawater (salinity 32 to 36 ‰ and temperature 28 ± 0.5°C) and fed twice daily with a commercial shrimp feed (CP # 4004, CP Feeds, Samut Sakorn, Thailand).

25.2.3 Digestibility tanks

The tanks used in the digestibility studies were circular, white polyethylene (100 L capacity, 600 mm diam.), fitted with a central standpipe drain. They were supplied with filtered (10 µm), heated seawater flowing at a rate of 500 mL min^{-1} to maintain tank temperatures at 29.0 ± 0.4°C, and with continuous aeration from a single air-stone. In-flowing seawater was used to create a gentle circular current within the tank to aid the concentration of waste in the centre.

25.2.4 Feed formulation and preparation

The reference diet used in this study (Table 25.3) was formulated to be nutritionally adequate and attractive to the shrimp, with 390 g/kg crude protein and 100 g/kg total lipid, on DM basis. Micronutrients were included at twice the minimum rate in the reference diet to ensure that they were not deficient when diluted with the test ingredients in the test diet formulations. The test diets comprised 50% by weight of the kernel meal ('as used' basis) and 50% by weight of the reference diet mash ('as used') (Table 25.3). The test diets had a similar crude protein content as the Reference diet (range: 380 to 425 g/kg) but slightly less total lipid (~ 90 g/kg). Ytterbium acetate tetrahydrate (99.9%, Aldrich, Sydney, Australia) was included in the feeds as an inert digestibility marker at a rate of 0.5 g/kg.

Water was added to the mixed ingredients to form a dough containing 40 to 50% moisture. The dough was extruded twice through a 3 mm die of a meat mincer (Hobart Corporation, Troy, OH, USA) to form spaghetti-like strands which were air dried in a forced-draught cabinet at 40°C, and then re-ground to pass through a 0.500 mm screen. Additional water was added to the re-ground material and the 'feed' mixed to form a dough again. This dough was extruded twice through the mincer, steamed for 5 min and air dried again before being broken-up into 5 to 10 mm pellets and stored at -5°C until used. This process was found to significantly improve the homogeneity of the feed pellets (Smith and Tabrett, 2004).

25.2.5 Experimental

The two digestibility experiments involved the feeding of the reference diet and six lupin kernel meal diets to groups of prawns (mean weight ± SD: Experiment 1 = 23.5 ± 3.8 g, Experiment 2 = 16.6 ± 2.4 g). In both experiments, six tanks, each containing two randomly selected prawns, were allocated to each dietary treatment. The prawns were placed in the tanks 7 days prior to

the start of the faecal collection periods, to adapt to their allocated diet. During the adaptation period the prawns were fed twice daily and no faeces were collected. After the adaptation period, and commencing on a Monday at 06:00 am, the prawns were fed every 6 h, with a 30 second interval between feeding successive tanks. Thirty minutes after the feed was put in the tanks, all the uneaten feed pellets and fragments were removed from the tanks by siphoning and discarded. Thereafter, faeces from individual tanks were collected by siphoning 3 h after feeding and again immediately before feeding. This process ran continuously for 5 d each week until Saturday mornings at 06:00 am. Between Saturday and Monday mornings, the prawns were fed twice daily and no faeces were collected.

The faeces siphoned from the each tank were collected into a 10 L bucket and within 30 min were transferred into a 10 mL centrifuge tube using a wide mouth pipette tip and bulb. The excess water was decanted from the centrifuge tubes after a short settling time. Distilled water was added to the tubes to make the volume up to 10 mL and the tubes centrifuged at 2000 rpm (700 rcf) for 30 sec. The supernatant was decanted off, and the tubes capped and placed in a freezer. Once frozen, the faecal pellet was transferred to a pre-weighed sample vial and stored at -20°C.

This routine was maintained for about 10 weeks in both experiments until at least 2 g dry weight of faecal material (~30 g of wet faeces) had been collected from each tank. This was the amount required for the intended chemical analyses for dry matter (DM), crude protein, energy and ytterbium, and additionally in the second experiment, for hydrolysis and amino acid determination. At the end of the experiment, faeces were freeze-dried, ground and stored at -20°C.

25.2.6 Chemical analyses

Samples of faeces, finely ground feed and lupin kernel meals were analysed using standard laboratory methods essentially in accordance with AOAC International (1999) recommendations. DM was determined gravimetrically after drying at 105°C to constant weight, generally for 16 h, and ash by heating and ignition at 600°C for 6 h. The total N content was determined using a modified Kjeldahl digestion (Bradstreet, 1965) followed by colorimetric analysis (Searle, 1984) in a Technicon segmented flow autoanalyser (Technicon Instruments Corporation, Tarrytown, NY, USA) (Varley, 1966). Crude protein (CP) was calculated by multiplying total N by 6.25. Total lipid was determined gravimetrically following extraction with chloroform-methanol (ratio 2:1) (Folch et al., 1957). The concentration of Yb was determined using a Varian Vista Pro axial CCD simultaneous ICP-OES (Varian Techtron, Mulgrave, Victoria, Australia) after digestion in nitric acid / perchloric acid mixture (McQuaker et al., 1979). Gross energy (GE) was determined by isothermal bomb calorimetry using a microprocessor-controlled Leco AC 200 automatic bomb calorimeter (Leco Corp. St Joseph, MI, USA). Amino acids, including methionine and cysteine, were determined after hydrolysis using 6M HCl with 0.5% phenol and DTDP for 24 h at 110 °C (Barkholt and Jensen, 1989). Amino acids were analysed by HPLC as the OPA and FMOC derivatives using a C18 column.

25.2.7 Calculations

The ADMD, ACPD, and ADE of the reference and test diets were calculated using the following equation:

$$AD_{N\ in\ Diet}(\%) = 100 * \left\{1 - \left(\frac{M_D * N_F}{M_F * N_D}\right)\right\}$$

where M_D and M_F is the concentration (on a DM basis) of the marker in the diet and faeces, respectively, and N_D and N_F are the concentration (on a DM basis) of the analyte of interest (nutrient, DM or energy) in the diet and faeces, respectively.

The test ingredient (lupin kernel meal) ADMD, ACPD and ADE were calculated using the respective digestibility of the test feed and of the reference diet in the equation described by Pfeffer et al. (1995):

$$AD_{NI} = \frac{1}{\alpha}\left[AD_{NTD} - (1-\alpha)\, AD_{NRD}\right]$$

where AD_{NI}, AD_{NTD} and AD_{NRD} are the apparent digestibility of the analyte in the ingredient, in the test diet and in the reference diet, respectively, and where α is the proportion of the analyte in the test diet that is contributed by the test ingredient:

$$\alpha = \frac{i \times DM_I \times N_I}{(i \times DM_I + (1-i) \times DM_{RD}) \times N_{TD}}$$

where ***i*** is the inclusion level of the test ingredient in the test diet (as mixed), DM_I and DM_{RD} are the DM (as mixed) of the test ingredient and reference diet, respectively. N_I is the concentration of the analyte in the test ingredient (DM basis), and N_{TD} is the concentration of the analyte in the test diet (DM basis).

The concentration of nitrogen-free extractives (NFE) in the kernel meals was derived to include fibre and all other carbohydrate material:

NFE = 1000 – (Ash + Crude Protein + Total lipid), on a g/kg DM basis.

The apparent digestible protein content of a kernel meal was calculated as:

AD protein content (g/kg) = ACPD (%) x CP (g/kg) / 100

Similarly, the total amount of the digestible amino acids was calculated as:

AD total amino acids (g/kg) = Σ (ADj (%) *AAj (g/kg) / 100

where j represents each of the amino acids.

25.2.8 Statistical analysis

Faecal samples from each tank were kept separate as replicate samples. Hence six estimates of AD were made for each diet and for each test ingredient. AD data were analysed for homogeneity using Bartlett's test for homogeneity of variances prior to analysis to ensure valid use of ANOVA. Differences in the apparent digestibility of DM, CP, energy and individual essential amino acids of the kernel meals derived from the lupin cultivars were analysed using a one-way analysis of variance. Data from the two experiments were combined and each sample of kernel meal was treated as an independent sample (Snedecor and Cochran, 1967). Combining the data for analysis was considered appropriate as there was no significant difference between experiments in the ADMD of the reference diet, or in its ACPD or ADE. The same reference diet was used

in both experiments providing the link between them. The means of AD, standard deviations and number of replicates of the reference diet for Experiment 1 and 2 were as follows: ADMD 74.59% (1.2450, 6) and 75.72% (0.9412, 6); ACPD 82.50% (0.6651, 6) and 82.36% (0.5412, 6); ADE 82.03% (0.8954, 6), 82.84% (0.6808, 6), respectively. Where the same cultivar was grown at Katanning and Wongan Hills (Kalya, Mandelup, Tanjil), the AD data were also analysed using a two-way analysis of variance to determine the influence of different growing conditions on the digestibility.

25.3 Results

There were significant differences in the ADMD, ACPD and ADE among the cultivars of *L. angustifolius* (Table 25.4). The AD of the older cultivar, Gungurru, was the highest of the of *L. angustifolius* cultivars. The mean ADMD of the new cultivars of *L. angustifolius* was 62.2% (range: 56.5% to 65.3%), while that of Gungurru was 66.3% (s.e.m. ± 0.95%). The mean ACPD of the new cultivars was 94.0% (range: 92.7% to 95.7%) while that of Gungurru was 96.8% (s.e.m. ± 0.48%) and the mean ADE of the new cultivars was 73.7% (range: 69.6% and 76.3%) with Gungurru at 77.2% (s.e.m. ± 0.72%). The ADMD and the ADE of *L. luteus* cv. Wodjil (70.0% and 79.9%, respectively) was significantly greater than that of all of the samples of *L. angustifolius,* though the ACPD was similar (93.8%) (Table 25.4).

Though overall there was not a significant difference in ADMD, ADCP and ADE between growing regions/conditions (location) or between cultivars, among the three cultivars that were grown at Katanning and Wongan Hills, there was a significant interaction between location and cultivar (Table 23.5). Kalya from Wongan Hills had greater ADs than the Kalya from Katanning, Tanjil from Wongan Hills had lower ADs than the Tanjil from Katanning, while Mandelup from Wongan Hills was not different from Mandelup from Katanning (Table 25.5).

There was a significant, inverse relationship ($P < 0.05$) between NFE content of the kernel meals and ADMD ($R^2 = 0.63$) and ADE ($R^2 = 0.66$), but it was not significant for ACPD ($R^2 = 0.07$) (Figure 25.1). The mean amino acid digestibility across cultivars and amino acids was about 93%. Arginine generally had the highest AD (mean = 98%) and cystine the lowest (mean = 86%), (Table 25.6). The LSD ($P = 0.05$) for the estimates of amino acid digestibility were about 0.5% except for methionine (2.4%), proline (1.6%) and tyrosine (1.9%). There was close agreement between average AD of all amino acids of a cultivar and it's ACPD ($Y = 0.9851X + 1.9507$; $R^2 = 0.98$). There was also a strong linear relationship between the amount of digestible protein in a kernel meal and the total amount of the digestible amino acids ($R^2 = 0.98$) (Figure 25.2).

25.4 Discussion

Formulating cost-effective feeds for the aquaculture industry, which have low inclusion levels of fishery-sourced feed ingredients such as fishmeal, relies on the provision of sound data showing the effectiveness of alternative protein sources. Information about the digestibility of these ingredients is a vital component of this data. In earlier digestibility and growth response studies with older cultivars of lupins, lupin kernel meals have been shown to be a useful protein source in feeds for both fish and prawns. The cultivars chosen for this study are currently the most widely grown lupin cultivars in Australia, representing > 80% of the national production.

The digestibility of the old cultivar of *L. angustifolius*, Gungurru determined in this study, was similar to that reported previously by Smith (1998): ADMD, 66.3% and 67%, respectively;

ACPD, 96.8% and 94%, respectively; and ADE, 77.2% and 71%, respectively. Studies with silver perch using the same cultivar, Gungurru, were also similar, with ADMD, ADCP and ADE values of: 68%, 100% and 74% respectively (Allan et al., 1998). However, the digestibility of the new cultivars were generally slightly lower than that determined for Gungurru in this study, though the values were generally similar to those reported for Gungurru in the earlier study (Smith 1998). The mean ADMD of the new cultivars was 62.2%; the ACPD was 94.0% and the ADE was 73.7%. Glencross and co-workers, working with rainbow trout, *O. mykiss*, have assessed the digestibility of 60 samples of lupin kernel meal derived from a range of new and old cultivars grown in different locations and growing seasons, including the kernel meals used in this study. Their results indicated that the digestibility of the lupin kernel protein could vary between 70% and 100% (Glencross et al. 2006). However, data identifying which cultivars had the low digestibility has not been reported at this stage. Such a large variation in the protein digestibility is in contrast with the observations of this study with *P. monodon* where the range in ACPD of all *L. angustifolius* cultivars was only 4.1%.

Generally, the ADs of the amino acids closely match the average ACPD of the cultivars in Experiment 2 (93.7%). The AD of arginine was consistently greater, having an average AD of 98% while cysteine had the lowest (86%). The variability in the estimates of AD of the amino acids tended to be strongly influenced by the markedly higher estimates of AD from the Kalya sample. There was also greatest variability in the estimates of AD of methionine. Whether this was an analytical issue or a feature of the methionine peptide linkages remains to be resolved. However, there appears to be a close relationship between the digestible crude protein content of the kernel meals and the digestible amino acid content (Figure 25.2), suggesting the robustness of this data. The general close equivalence of ACPD and average AD of amino acids seen in this study was also noted by Akiyama et al. (1989), who reported that with the Pacific white shrimp *Penaeus (=Litopenaeus) vannamei*, the average digestibility of amino acids in a soybean meal test diet was ~90% while that of crude protein was 89.9%. However, Akiyama et al. (1989) did not report on the AD of methionine or cysteine. In a study with silver perch, Allan et al. (2000) found the digestibility of amino acids in *L. angustifolius* whole seed meal was high with an average apparent digestibility of about 98%. As with the current study, they also found that cysteine had the lowest apparent digestibility (79.5%). Again, the reason for this low digestibility has not been explained.

The NFE component in the kernel meals comprises mainly carbohydrates. The carbohydrate is comprised predominantly of soluble and insoluble non-starch polysaccharides (oligosaccharides and dietary fibre, respectively) and negligible amounts of starch (reviewed by van Barneveld, 1999). The non-starch polysaccharides, such as dietary fibre, are poorly digested by monogastric animals (van Barneveld, 1999), fish (Glencross et al., 2003), prawns (Akiyama, et al. 1989; Smith, 2002) and freshwater shrimp (González-Péna et al., 2002). As the protein in the kernel meal is highly digestible, and as the lipid is also likely to be highly digestible (Merican and Shim, 1995; Glencross et al. 2002), the relatively low ADMD of the kernel meals (56.5% to 70.0%) is a reflection of the low digestibility of the NFE. This appears to be supported by the significant trend of decreasing ADMD with increasing NFE content (Figure 25.1). It is interesting to note that the NFE did not appear to have any affect on ACPD (Figure 23.1). However, the concentration range of NFE in the samples of *L. angustifolius* was quite narrow: 404 g/kg to 469 g/kg (as used), and this would also have been reflected in the NFE content of the test diets.

To estimate the AD of the NFE, the difference between the digestible energy (ADE x GE/100) of the kernel meals and the calculated DE derived from crude protein and total lipid was calculated.

As the digestibility of lipid was not measured in this study, nor was it separated into its lipid classes, several assumptions were made: (a) total lipid in lupin kernel meal was comprised of both triacylglycerides (67%) and phospholipids (33%) (van Barneveld, 1999) and (b) that the digestibility of the triacylglycerides was 98% and phospholipids was 64% (Merican and Shim, 1995), giving a total lipid digestibility of 87%. The estimates of DE from NFE were found to be between 1.0 and 3.2 MJ $kg^{-1}$of kernel meal (Table 25.7). It is interesting to note that the lowest value was for *L. luteus* cv. Wodjil. Assuming the NFE was all carbohydrate, and using a conversion factor of 17.2 MJ kg^{-1} for carbohydrate (Cho et al., 1982), this equates to the energy provided by between 59 and 187 g of carbohydrate. Using these estimates, the AD of the NFE in *L. angustifolius* cultivars was calculated to vary between 22% and 41% (mean ± s.e. = 31% ± 1.4%), while that of *L. luteus* cv. Wodjil was 19%. In *L. luteus* cultivar Wodjil, the NFE content and its AD appear to differ quite markedly from the *L. angustifolius* cultivars, suggesting its NFE composition might be substantially different.

The method for calculating ingredient digestibility has been the subject of discussion in recent literature (Forster, 1996; Bureau et al., 1999) and a Letter to the Editor of Aquaculture 2006, 252, 103-105 (Bureau and Hua). The equation that we have used is based on that reported by Pfeffer et al. (1995). All these equations are equivalent as they are derived from the same base equation proposed by Kleiber (1975). The difficulty in using them appears to be in incorporating into the calculation the contribution of the test ingredient to the concentration of a particular nutrient (or analyte) in the test diet. To be able to do this, the DM content of the test ingredient and of the mixed ingredients (mash) of the reference diet need to be known. Another difference between the equation reported by Pfeffer et al. (1995) and the one advocated by Bureau and Hua (2006) lies in the use of either of two alternative parameters: the concentration of nutrient in the test diet (Pfeffer et al. 1995) or the concentration of nutrient in the reference diet (Bureau and Hua, 2006). Any differences in the estimates of ingredient AD are due to the errors inherent in the analysis of either of these diets. In addition to calculating the ADs using Pfeffer's equation, we have also calculated the ADs using the equations proposed by Forster (1996) and by Bureau and Hua (2006), and we have obtained closely similar results.

In conclusion, there were a number of significant differences among the ADs of the new cultivars of *L. angustifolius* when used in diets for *P. monodon.* However, their values were broadly similar and similar to the AD reported for the older cultivar, Gungurru. The ACPD was uniformly high, with the average of 94.3% across 12 samples, and the AD of the amino acids was of a similar value. The sulphur amino acid methionine, showed the most variability, with most of the variability due to the AD of one particular cultivar (Kalya). Whether this was a hydrolysis/analytical artefact or a feature of the methionine peptide linkages remains to be resolved. The AD of cysteine, another of the sulphur amino acids, was the lowest of the amino acids at 86%. The general consistency of the AD results across the range of cultivars, which represent over 80% of the production of narrow leafed lupins in Australia, suggest that nutritionists and feed formulators can confidently use mean AD values for dry matter, protein and energy for kernel meals comprising random mixtures of the new cultivars.

25.5 References

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Tables and Figures

Table 25.1 Proximate composition (g/kg DM), unless otherwise stated) of lupin kernel meals evaluated in the digestibility experiments.

Lupin cultivar[1]	Moisture	Ash	Crude protein	Total lipid	NFE	Energy (MJ/kg DM)
Experiment 1						
Kalya (KT)	101	36	418	96	451	20.7
Mandelup KT)	101	30	416	94	456	20.7
Tanjil (KT)	101	30	413	105	449	21.1
Myallie (KT)	102	31	453	89	422	20.8
Walan 2173(KT)	103	31	458	94	412	20.9
Gungurru (KT)	85	29	463	94	415	21.0
Experiment 2						
Kalya (WH)	71	37	494	80	389	20.6
Mandelup (WH)	69	34	468	81	416	20.6
Tanjil (WH)	63	34	480	88	398	20.6
Wonga (WH)	66	33	470	88	409	20.6
Myallie (CO)	83	37	426	87	450	21.1
Wodjil (CO)	73	44	546	94	316	20.4

[1] All kernel meals were from cultivars of *L. angustifolius*, except for Wodjil which is a cultivar of *L. luteus*. The region in Western Australia where the lupins were grown is indicated in parentheses after the cultivar name:WH = Wongan Hills, KT = Katanning, CO = Coorow.

Table 25.2 Amino acid composition of lupin kernel meals (g/kg DM) evaluated in Experiment 2 of the digestibility study (WH = Wongan Hills; CO = Coorow).

Amino acid	Kalya (WH)	Mandelup (WH)	Tanjil (WH)	Wonga (WH)	Myallie (CO)	Wodjil (CO)
Alanine	1.63	1.64	1.54	1.49	1.49	1.84
Arginine	5.92	5.20	5.44	5.40	4.66	6.11
Aspartic acid	5.52	5.06	5.19	5.06	4.54	5.85
Cysteine*	0.81	0.80	0.70	0.66	1.86	3.90
Glutamic acid	11.50	10.11	10.44	10.27	9.08	13.45
Glycine	1.95	1.95	1.83	1.79	1.70	2.16
Histidine	1.06	0.86	0.96	0.86	1.03	1.39
Isoleucine	1.90	1.80	1.76	1.85	1.60	2.00
Leucine	3.58	3.31	3.23	3.30	2.88	4.26
Lysine	2.29	2.21	2.00	2.14	1.24	1.68
Methionine	0.22	0.33	0.27	0.28	0.20	0.30
Phenylalanine	1.86	1.79	1.77	1.81	1.58	2.08
Proline	3.08	3.26	3.71	2.40	2.56	3.62
Serine	2.59	2.31	2.42	2.36	2.15	2.86
Taurine	0.00	0.00	0.00	0.00	0.00	0.00
Threonine	1.75	1.75	1.67	1.58	1.57	1.92
Tyrosine	1.88	1.77	1.82	1.80	1.70	1.68
Valine	1.70	1.80	1.66	1.68	1.58	1.86

* Determined as cysteic acid from conversion of each molecule of cysteine to one molecule of cysteic acid and each molecule of cystine to two molecules of cysteic acid.

Table 25.3 Ingredient composition (g/kg 'as used') of reference diet and test diets used in the digestibility experiments.

Ingredient	Reference diet	Test diets
Lupin kernel meal	–	500
Flour (wheat)[1]	376	188
Gluten (Wheat, 76% CP) [2]	120	60
Fishmeal (Peruvian 68% CP) [3]	200	100
Squid meal [3]	100	50
Crustacean meal [4]	100	50
Cod liver oil[5]	40	20
Soybean lecithin (70% lipid)[6]	30	15
Cholesterol (100%)[7]	10	5
Vitamin mix[8]	20	10
Sodium ascorbyl-2-polyphosphate (Stay C)[9]	2	1
Astaxanthin (Carophyll Pink 10%)[10]	1	0.5
Ethoxyquin (Banox E) [9]	0.4	0.2
Ytterbium acetate tetrahydrate[11]	0.5	0.5

[1] Flour, White Wings, Brisbane, Queensland, Australia

[2] Wheat gluten (76% CP), Janbak Industries Pty Ltd, Brisbane, Queensland.

[3] Fishmeal and squid meal supplied by Ridley Aquafeeds Pty Ltd, Narangba, Queensland

[2] Corn starch, Janbak Industries Pty Ltd, Brisbane, Queensland, Australia.

[4] Crustacean meal, Inual, Santiago, Chile, supplied by Ridley Aquafeeds

[5] Melrose Laboratories, Box Hill, Victoria, Australia.

[6] Supplied by Janbak Industries Pty Ltd, Brisbane, Queensland.

[7] Ajax Chemicals, Sydney, NSW, Australia

[8] Vitamin mix. (Conklin,1997), supplied by Rabar Pty Ltd, Beaudesert, Queensland

[9] Adisseo Australia, Carole Park, Qld

[10] Donated by DSM Nutritional Products Australia Pty Ltd, Sydney, NSW.

[11] Aldrich, Sydney NSW.

Table 25.4 Derived apparent digestibility (%) of dry matter (ADMD), crude protein (ACPD) and energy (ADE) of lupin kernel for the black tiger prawn, *P. monodon.*

Lupin Cultivar	ADMD [2]	ACPD [2]	ADE [2]
Kalya (KT)	59.6 [e]	93.0 [d]	71.9 [e]
Mandelup KT)	60.8 [de]	93.8 [cd]	72.2 [e]
Tanjil (KT)	65.3 [b]	95.5 [ab]	76.3 [bc]
Myallie (KT)	64.3 [bc]	95.7 [ab]	75.0 [cd]
Walan 2173(KT)	62.5 [cd]	94.1 [cd]	73.8 [de]
Gungurru (KT)	66.3 [b]	96.8 [a]	77.2 [b]
Kalya (WH)	64.6 [bc]	95.0 [bc]	75.8 [bc]
Mandelup (WH)	62.4 [cd]	93.3 [d]	73.4 [de]
Tanjil (WH)	61.3 [de]	92.8 [d]	72.8 [e]
Wonga (WH)	64.9 [bc]	94.6 [bc]	75.9 [bc]
Myallie (CO)	56.5 [f]	92.7 [e]	69.6 [f]
Wodjil (CO) [1]	70.0 [a]	93.8 [cd]	79.9 [a]
± s.e.m	± 0.95	± 0.48	± 0.72

[1] Cultivar of *L. luteus*, all other cultivars are of *L. angustifolius*. The agricultural region in Western Australia where the lupins were grown is indicated in parentheses after the cultivar name: WH = Wongan Hills, KT = Katanning, CO = Coorow.

[2] Means (n = 6) not sharing a common superscript within a column are significantly different ($P < 0.05$).

s.e.m. = standard error of the mean

Table 25.5 Comparison of apparent digestibility data for three cultivars of *L. angustifolius* grown at two locations, Katanning and Wongan Hills.

Location	Kalya	Mandelup	Tanjil	Mean
Apparent dry matter digestibility (%) [1]				
Katanning	59.6 [b]	60.8 [b]	65.3 [a]	61.9
Wongan Hills	64.6 [a]	62.3 [ab]	61.3 [b]	62.8
Mean	62.1	61.6	63.3	(± 1.03)*
Apparent crude protein digestibility (%) [2]				
Katanning	93.0 [b]	93.8 [bc]	95.5 [a]	94.1
Wongan Hills	95.0 [ab]	93.3 [c]	92.8 [c]	93.7
Mean	94.0	93.5	94.1	(± 0.42)*
Apparent digestibility of energy (%) [3]				
Katanning	71.9 [b]	72.2 [b]	76.3 [a]	73.4
Wongan Hills	75.8 [a]	73.4 [b]	72.8 [b]	74.0
Mean	73.9	72.9	74.6	(± 0.74)*

[1, 2, 3] values with the same superscript letter do not differ significantly ($P = 0.05$)

* ± standard error of the mean; refers to (cultivar x location) interaction term.

Table 25.6 Apparent digestibility (%) of amino acids of kernel meals from cultivars of narrow leafed lupin (*Lupinus angustifolius)* and yellow lupin (*L. luteus* cv. Wodjil) used in Experiment 2 of the digestibility study**.

Amino acid	Kalya (WH)	Mandelup (WH)	Tanjil (WH)	Wonga (WH)	Myallie (CO)	Wodjil (CO)	± s.e.m.
Alanine	93	93	92	94	90	90	0.6
Arginine	99	98	98	98	97	96	0.6
Aspartic acid	95	92	92	94	91	92	0.4
Cysteine*	94	86	87	90	79	80	0.7
Glutamic acid	98	97	97	97	96	96	0.2
Glycine	93	93	91	94	90	89	0.6
Histidine	96	96	93	98	91	92	0.6
Isoleucine	97	95	95	95	92	93	0.4
Leucine	96	94	94	94	93	94	0.4
Lysine	93	90	95	92	91	93	0.5
Methionine	100	86	88	92	88	90	2.4
Phenylalanine	95	94	95	96	94	94	0.5
Proline	100	94	96	96	90	90	1.4
Serine	95	92	92	94	91	92	0.4
Threonine	92	91	89	91	87	88	0.6
Tyrosine	98	95	94	93	93	94	1.8
Valine	94	91	92	93	91	90	0.5

* Determined as cysteic acid from conversion of each molecule of cysteine to one molecule of cysteic acid and each molecule of cystine to two molecules of cysteic acid.

The agricultural region in Western Australia where the lupins were grown is indicated in parentheses below the cultivar name: WH = Wongan Hills, KT = Katanning, CO = Coorow.

Table 25.7 Analysed and calculated gross energy (GE) and digestible energy (DE) of lupin kernel meals (LKM) evaluated in the digestibility experiments. Energy is on reported on a MJ/kg DM basis.

Lupin cultivar[1]	NFE (g/kg)	Determined DE of LKM	Calculated DE from CP+lipid[2]	DE of NFE (difference)	Digestible NFE (g/kg)	AD of NFE (%)
Experiment 1						
Kalya (KT)	451	14.9	12.5	2.4	141	31
Mandelup KT)	456	15.0	12.4	2.5	147	32
Tanjil (KT)	449	16.1	12.9	3.2	187	41
Myallie (KT)	422	15.6	13.3	2.3	136	32
Walan 2173(KT)	412	15.4	13.4	2.1	119	29
Gungurru (KT)	415	16.2	13.8	2.4	141	34
Experiment 2						
Kalya (WH)	389	15.6	13.8	1.8	107	27
Mandelup (WH)	416	15.1	13.1	2.1	120	29
Tanjil (WH)	398	15.0	13.5	1.5	87	22
Wonga (WH)	409	15.6	13.5	2.1	124	30
Myallie (CO)	450	14.7	12.3	2.4	139	31
Wodjil (CO)	316	16.3	15.3	1.0	59	19

[1] All kernel meals were from cultivars of *L. angustifolius*, except for Wodjil that is a cultivar of *L. luteus*. The agricultural region in Western Australia where the lupins were grown is indicated in parentheses after the cultivar name: WH = Wongan Hills, KT = Katanning, CO = Coorow.

[2] Calculated values are based on the following energy values: protein, 23.4 MJ/kg; lipid, 39.8 MJ/kg; NFE (= carbohydrate), 17.2 MJ/kg (Cho et al., 1982), and assumed AD of lipid = 87% (Merican and Shim, 1995).

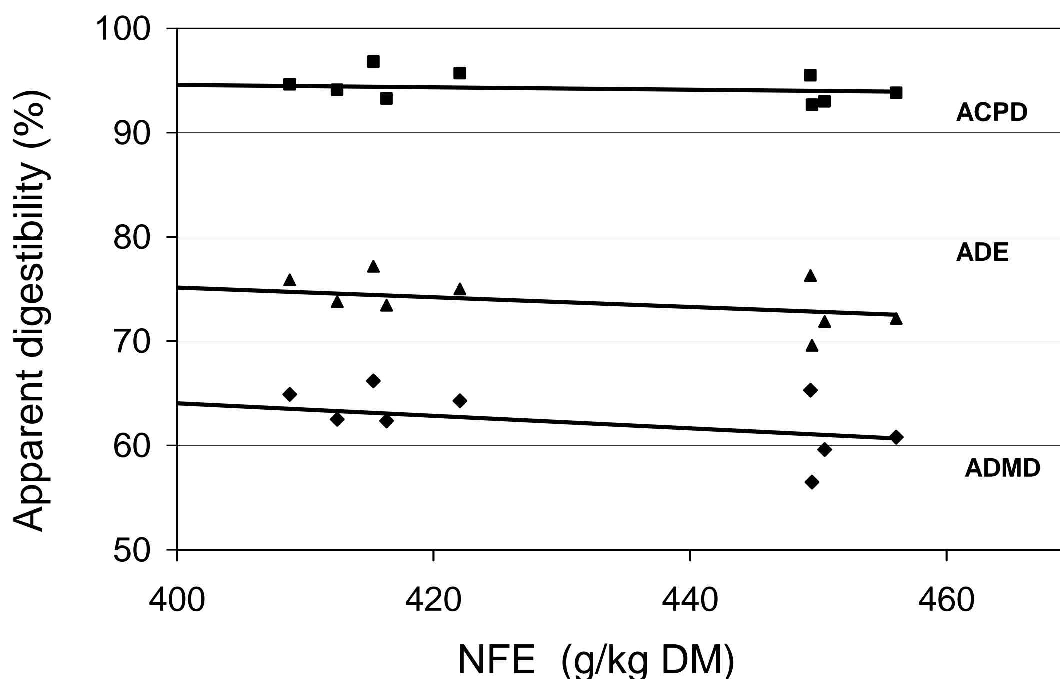

Figure 25.1 Relationship between nitrogen-free extractives (NFE) and apparent digestibility of dry matter (ADMD), crude protein (ACPD) and energy (ADE) of lupin cultivars determined with black tiger prawns, *P. monodon*.

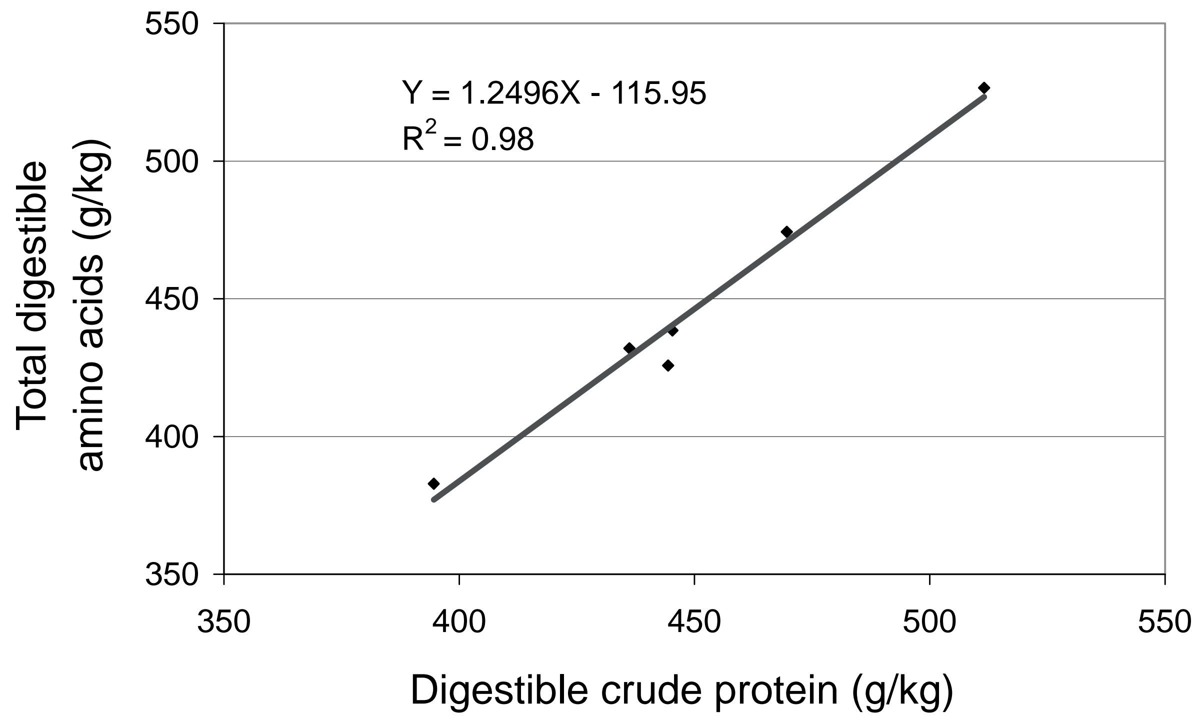

Figure 25.2 Relationship between digestible crude protein of lupin kernel meals (g/kg) (X) and the sum of digestible amino acids (g/kg) (Y).

26.0 Growth response of the black tiger shrimp, *Penaeus monodon* fed diets containing different lupin cultivars[a]

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Abstract

Over the last ten years, new cultivars of lupins have been developed by plant breeders which have largely replaced the cultivars that were studied in previous research with shrimp. There was a need to establish if the breeding programs had introduced changes in the new lupin cultivars that would affect the nutritional value of the kernel meal for shrimp. We have determined the performance of seven of the new cultivars of *Lupinus angustifolius* when used to replace fishmeal in diets for the black tiger shrimp, *Penaeus monodon*. The *L. angustifolius* cultivars represent about 80% of Australia's lupin production. We have also compared the performance of the new cultivars with that of solvent-extracted soybean meal. Three 50-d growth response experiments were carried out using an array of 100 L circular aquarium tanks in an open seawater system. Six replicate tanks each stocked with five juvenile shrimp were assigned to each treatment in completely randomised design experiments. Lupin kernel meal and solvent-extracted soybean meal was used to replace fishmeal in the experimental diets on an iso-nitrogenous basis. The diets contained 454 g/kg or 420 g/kg of crude protein (on a dry matter basis), with the plant proteins usually contributing 41.5% of the dietary protein. In all three experiments the growth rate of shrimp fed the diets containing lupin kernel meal or soybean meal was as good as, or better than, that obtained with the basal diet. Survival in all experiments was high (mean ~90%). FCRs were variable and high, reflecting the difficulty of not feeding to excess in small aquarium systems, but there was generally little difference between the FCR of the basal diet and that of the lupin kernel meal or soybean meal diets. This study has demonstrated that lupin kernel meal can be used to replace at least 40 % of the fishmeal protein in diets for *P. monodon*, and that the new cultivars perform equally to solvent-extracted soybean meal when used on a protein-equivalent basis. From the amino acid analysis of the diets used in the experiments, it appears that that the reported requirements of juvenile *P. monodon* for methionine significantly overestimate the true requirements. Further clarification of this issue is warranted, as it is possible that formulators are restricting the inclusion level of lupins in shrimp feeds in order that they meet the reported requirement for methionine.

26.1 Introduction

Much of the recent increase in global aquaculture production has been brought about through the adoption of intensive farming practices using formulated feeds. Feeds used in the culture of carnivorous fish and crustaceans generally contain a high concentration of protein, much of which is presently obtained through the inclusion of fishmeal at between 200 and 300 g kg^{-1} of feed (Tacon, 2002). In 2001, the feeding of these species required an estimated 16.7

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million tonnes of aquafeeds, containing about 2.6 million tonnes of fishmeal (or 43.1% of the total global production) (FIN, 2004). However, world fishmeal production has remained relatively static at 6.2 million tonnes (IFFO, 2006) and is unlikely to increase further. Fishmeal production is also subject to sharp, periodic declines such as in 1998 when only 4.75 million tonnes were produced (Barlow, 2002). It is evident from these statistics that continued expansion of aquaculture will not be possible if fishmeal is relied upon as the main source of protein in aquafeeds. Moreover, competition for the raw materials of fishmeal for processing for human consumption and for conversion to fishmeal will increase. Likewise, demand for fishmeal from other feed industry sectors such as the pig, poultry and pet food industries will increase. These issues will force fishmeal prices up until its usage in aquafeeds becomes uneconomical. In any event, if aquaculture is to become a net and increasing contributor to human food supplies, it is critical that aquafeeds become less reliant on fishmeal.

There has been a considerable amount of research evaluating alternative, terrestrial protein sources for use in aquaculture feeds (Lim et al., 2007). Much of the research interest has been directed towards the use of soybean meal, but more recent studies have extended to the use of field peas, canola and lupins. The nutritional value of a number of species and cultivars of lupins has been assessed for a wide variety of fish and shrimp species (reviewed by Glencross, 2001, Smith et al., 2007a). Lupins appear to be useful, protein-rich ingredients that can partially replace fishmeal in feeds for both fish and shrimp (Hughes, 1991; Burel et al., 1998; Smith et al., 2000). As Australia contributes about 80% of the global production of lupins, there has been a significant research effort in Australia to evaluate lupin products in aquaculture feeds (Allan and Rowland, 1998; Smith, 1998; Carter and Hauler, 2000). Lupin kernel meal was found to be a better feed ingredient than the whole seed meal, as the removal of the seed coat resulted in a much more digestible product with an increased protein content (Smith, 1998; Booth et al., 2001; Glencross, 2001).

Much of the early research was carried out using kernel meals derived from the narrow leafed lupin, *Lupinus angustifolius,* particularly a variety (or cultivar) called Gungurru. During the 1990's, Gungurru was the most widely grown cultivar in Australia. Since then, lupin-breeding programs have produced new cultivars that are better suited to particular soil types and climatic conditions found in the different growing regions. Gungurru has been largely replaced by these new cultivars and now represents < 5% of Australian production (B. Buirchell, WA Agriculture. pers. comm.; Pulse Australia, 2006). Glencross and co-workers have determined the digestibility of the new kernel meals when used in diets for rainbow trout, *Oncorhynchus mykiss* (Glencross et al., 2003; Glencross and Hawkins, 2004), and Smith and co-workers determine the digestibility of the kernel meals in diets for the black tiger shrimp, *Penaeus monodon* (Smith et al., 2007). However, there does not appear to be any comparative growth response data demonstrating the effect of inclusion of these kernel meals in feeds for any species of fish or shrimp.

In this study with *P. monodon,* we have carried out two experiments to determine the growth response and feed conversion ratio (FCR) of diets containing relatively high inclusion levels of a number of the new cultivars of *L. angustifolius* that have been grown under two different growing conditions. These cultivars represent about 80% of Australia's current lupin production. We have also compared the response of the shrimp to diets containing solvent-extracted soybean meal at two inclusion levels, with diets containing protein-equivalent inclusions of two samples of lupin kernel meal.

26.2 Materials and methods

26.2.1 Lupin kernel meals

Samples of whole seed of *L. angustifolius* lupins were obtained from the lupin breeding program of the Department of Agriculture in Western Australia. The lupins were grown at either of two of the Department's research field stations, Katanning (33.69 S, 117.61 E) and Wongan Hills (30.89 S, 116.72 E) (Table 26.1). Both batches of seed were obtained from the 2003 crop. The harvested seed was segregated by source and cultivar and stored at 4°C prior to processing (Table 26.1). An additional sample of seed comprising a mixture of *L. angustifolius* cultivars was provided by a grain exporting company (Cooperative Bulk Handling, Forrestfield, Western Australia). This sample was considered to be typical of the product that would be commercially available on the international market. Solvent-extracted soybean meal was included in the study and was provided by a commercial feed company (Ridley AquaFeeds Pty Ltd, Narangba, Qld. Australia) (Table 26.1).

Seed of the different lupin cultivars were segregated during processing. The seeds of each cultivar were separated according to size using round-holed 7mm, 6mm and 5mm sieves. The size fractions were separately split using a disc-mill dehulling unit (Department of Agriculture, South Perth, WA, Australia). The fractions of split (or dehulled) material were then re-combined, and the hulls separated from the kernels using air stream mediated density classification. Any remaining seed hull fragments were manually removed to ensure a 100% pure preparation of seed kernels of each variety. The kernels were then rotor-milled (Retsch, Haan, Germany) through a 750 µm screen. Samples were analysed to determine their proximate composition (Table 26.1) and the amino acid composition of a sub-set of the samples (Table 26.2).

26.2.2 Experimental design

Three growth response experiments were carried out to evaluate the lupin kernel meals in shrimp feeds. In each experiment there were seven or eight treatments: a basal diet containing no lupin kernel meal, a high-performing reference diet and 5 or 6 test diets containing lupin kernel meal or soybean meal (Tables 26.3, 26.4 and 26.5). The reference diet was a shrimp feed formulated for *Penaeus japonicus* that contained 60% crude protein on an 'as used basis' (Lucky Star, Hung Kuo Industrial Co, I-Lan, Taiwan). A total of seven cultivars of *L. angustifolius* and the commercial mixture of cultivars were evaluated in the study. Each experiment was run for 50 days with 6 replicate tanks assigned to each treatment in a complete randomised design.

The first experiment was used to assess cultivars that had been grown in the south of the Western Australian wheat belt, at Katanning. These cultivars were Belara, Kalya, Mandelup, Tanjil, Walan 2173 and Myallie (Table 26.3). The second experiment examined kernel meals from lupins grown in the northern growing areas of the wheat belt, at Wongan Hills. These were the cultivars Kalya, Mandelup, Tanjil and Wonga (Table 26.4). The cultivar Myallie that had been used in the first experiment was also included in this experiment. The third experiment compared diets containing solvent extracted soybean meal at two inclusions levels (~190 g/kg and ~ 330 g/kg) with diets containing iso-nitrogenous inclusions of kernel meals from the cultivar Kalya from Katanning, and from the mixture of cultivars provided by Cooperative Bulk Handling (CBH Mixed) (Table 26.5).

26.2.3 Diet preparation

Diets for each of three experiments were prepared separately just prior to the start of the experiment (Table 26.3, 26.4 and 26.5). Before being weighed out, dry ingredients were sieved and ground to ensure all of the material passed through a 710 µm screen. The weighed ingredients (Table 26.6) were thoroughly mixed in a planetary mixer before a volume of water equivalent to approximately 40% of the dry weight of ingredients was added, and mixed further to form a crumbly dough. The dough was extruded through the meat grinder attachment of a Hobart mixer (Hobart Corporation, Troy, OH, USA). The extruded, spaghetti-like strands (~3 mm diameter) were steamed for 5 min in a steamer at atmospheric pressure (Curtin & Son, Sydney, Australia), air-dried overnight in a forced-draught cabinet at 40°C and broken into pellets 5 to 8 mm long. The pellets were stored at -20°C until used.

26.2.4 Experimental animals and tanks

Juvenile *P. monodon*, were obtained from commercial shrimp farms in northern Queensland, Australia. They were held at the CSIRO Marine Research Laboratory, Cleveland in 2500 L tanks for about one week before being transferred to the smaller tanks used for the growth response experiments. While held in the 2500 L tanks, the shrimp were fed twice daily with a commercial *P. monodon* feed (CP # 4004, CP Feeds, Samut Sakorn, Thailand). The tanks were supplied with flow-through seawater (salinity 32 to 36 ‰) that maintained the temperature at 28 ± 0.5°C. For the growth response experiments, an array of circular, white polyethylene indoor tanks (120 L capacity, 600 mm diam.) was used. Each tank was supplied with filtered (10 µm), heated seawater flowing at a rate of 500 mL min^{-1} to maintain tank temperatures at 29.0 ± 0.5°C, and provided with supplementary aeration from a single air-stone. A 12 h light: 12 h dark photoperiod was maintained throughout the experiments. Water temperatures and salinities were monitored daily.

26.2.5 Experimental management

Prior to the start of an experiment, the shrimp were individually weighed and sorted into size classes, so that shrimp within a class was had a weight range of 0.25 or 0.5 g. One or more size classes were selected for the experiment, so as to minimise the weight range of the shrimp. Shrimp of less than 3 g were not included in any of the experiments. The shrimp were distributed among the array of tanks with six shrimp in each tank, such that the biomass in all the tanks was similar. The shrimp were allowed to adapt to the tank conditions and the basal diet for between 5 and 7 days before they were individually weighed again at the start of the experiment. At this weighing, only five shrimp were returned to each tank to further reduce the variability in the weight range of individual shrimp and the biomass among tanks. They were weighed again after 25 d and at the end of the experiment at 50 d. During the experiment, the tanks of shrimp were fed weighed allocations of their assigned feeds twice daily, nominally at 0830 and 1700 h. The tanks were cleaned daily in the afternoon and the amount of uneaten feed in the tanks was noted using a scale of 0 to 4. The following day's allocation of feed was adjusted according to this value, so as to minimise the amount of uneaten feed but to ensure that growth was not limited by consistent underfeeding. The incidence of any dead or missing shrimp was also noted during the tank cleaning and the dead or missing shrimp were replaced within 24 h with tagged shrimp of similar size. Tagged replacement shrimp were used to maintain a constant stocking density in the tanks but were not included in the data used in the analysis of growth rates or survival. Though individual weights were recorded, only the mean weight of untagged shrimp within each tank was used in the data analysis.

26.2.6 Statistical analysis

The mean value from each tank for each response parameter (initial weight, final weight, growth rate, daily growth coefficient, feed allocation, FCR, survival) was the statistical unit for the data analysis. Differences across treatments of means of the response parameters were tested using one-way ANOVA in accordance with the design of each experiment. Differences between treatment effects were examined *a-posteriorly* using Fischer's protected '*t*' test (Steel and Torrie, 1980) wherein differences between means were examined only where the '*F*' test of the ANOVA was significant ($P < 0.05$).

26.2.7 Chemical analyses

Samples of finely ground feed and lupin kernel meals were analysed using standard laboratory methods essentially in accordance with AOAC International (1999) recommendations. Dry matter (DM) was determined gravimetrically after drying at 105°C to constant weight, generally for 16 h, and ash by heating and ignition at 600°C for 6 h. The total N content was determined using a modified Kjeldahl digestion (Bradstreet, 1965) followed by colorimetric analysis (Searle, 1984) in a Technicon segmented flow autoanalyser (Technicon Instruments Corporation, Tarrytown, NY, USA) (Varley, 1966). Crude protein (CP) was calculated by multiplying total N by 6.25. Total lipid was determined gravimetrically following extraction with chloroform-methanol (ratio 2:1) (Folch et al., 1957). Gross energy (GE) was determined by isothermal bomb calorimetry using a microprocessor-controlled Leco AC 200 automatic bomb calorimeter (Leco Corp. St Joseph, MI, USA). Amino acids, including methionine and cysteine, were determined after hydrolysis using 6M HCl with 0.5% phenol and DTDP for 24 h at 110°C (Barkholt and Jensen, 1989). This hydrolysis procedure converts cysteine and cystine to cysteic acid in which form they were analysed. Amino acids were analysed by HPLC as the OPA and FMOC derivatives using a C18 column.

26.3 Results

26.3.1 Experiment 1

The growth rate (g/wk) and daily growth coefficient (DGC, % d^{-1}) of the shrimp fed the Reference diet was significantly greater than that of all other treatments. Shrimp fed the Kalya (KT) diet had significantly higher growth rates than those fed the Tanjil (KT) diet. However, there were no significant differences among the other treatments (Table 26.9). When the data set was analysed without the Reference data (*a priori* expectation of the higher performance with this high-protein feed), there were no significant differences among the treatments. Feed allocation was highly variable with the greatest amount of feed being allocated with the Kalya (KT) treatment and the lowest with the Basal 1 diet. FCR's were high (range 3.5 to 7.1) and variable with the lowest FCR obtained with the Reference diet. Average survival was 95% and not significantly different among treatments.

26.3.2 Experiment 2

The growth rate (g/wk) and daily growth coefficient (DGC, % /d) of the shrimp fed the Reference diet was significantly greater than that of all other treatments. However, there were no significant differences among any of the other treatments (Table 26.10). Feed allocation was greatest with the Reference diet but did not differ among the basal diet and lupin-containing diets. There were no significant differences among treatments in FCR. The lowest FCR was obtained with the

Reference diet (2.7) and the highest with the basal diet (4.0), whereas the FCR's obtained with the lupin-containing diets were similar (range 3.0 to 3.2). Average survival was 89% and did not differ significantly among treatments.

26.3.3 Experiment 3

The growth rate (g/wk) and daily growth coefficient (DGC, % /d) of the shrimp fed the Reference diet was significantly greater than that of all other treatments. There were no significant differences in growth rate (g/wk) among any of the other treatments (Table 26.11). However, the DGC of shrimp fed the diet containing Kalya (KT) at the moderate inclusion level was significantly greater than that of shrimp fed the basal diet. Feed allocation was variable with the greatest amount of feed allocated to shrimp fed the Reference diet. The data was analysed to establish if there was a significant effect of inclusion level of soybean meal or lupin kernel meal on feed allocation. The analysis showed a significant interaction between grain type and inclusion level, though there was significantly more feed containing the higher inclusion level allocated with soybean meal and with the CBH Mixed lupin kernel meal. FRC's were variable and there was no trend that could be associated with treatment. The average survival across all treatments was 89%. However, survival was significantly greater (100%) with the Reference diet and the diet containing Kalya (KT) at a high inclusion level, and lowest with CBH Mixed (moderate inclusion level) (77%) and soybean meal (moderate inclusion level) (80%). There was no apparent trend associated with treatment or inclusion level. However, when the data set was analysed without the Reference diet data, just including treatments fed diets with the same nutrient specifications, there were no significant differences among treatments.

26.4 Discussion

In all three experiments, shrimp fed diets containing lupin kernel meal performed as well as, or better than, shrimp fed the respective basal diet. This is of particular note as the inclusion level of the kernel meal in most of the feeds was high-varying between 450 and 523 g/kg in Experiment 1, between 351 and 398 g/kg in Experiment 2, and from 396 to 428 g/kg at the higher inclusion levels in Experiment 3. In the three experiments this constituted about 41.5% of the crude protein in the diet. As the apparent digestibility of crude protein in the kernel meals was similar, about 94% (Smith et al. 2007b), they are calculated to have contributed approximately 43% of the digestible protein in the diets. In all three experiments, the growth rate and DGC of shrimp fed the Reference diet was significantly greater than that of shrimp fed the other treatments. This was expected as the Reference diet is a high-cost feed that is formulated for *P. japonicus*. It has higher nutrient specifications, particularly protein (600 g/kg as used), than those recommended for *P. monodon.* In a previous study with *P. monodon,* where this feed has been used as a reference diet, we have observed the same superior performance (Williams et al., 2005).

In a series of studies with the kernel meal from another species of lupins, the white lupin, *L. albus,* Sudaryono and co-workers showed that the growth rate of juvenile *P. monodon* decreased markedly when 300 g/kg and 400 g/kg of the kernel meal was used to replace 75% and 100% of the fishmeal in the basal diet (Sudaryono et al., 1999a). In previous studies where kernel meal from the older and now largely superseded cultivar of *L. angustifolius*, Gungurru, had been used to replace fishmeal in diets for *P. monodon,* a significant reduction in growth rate was observed when the inclusion level of the kernel meal was greater than 250 g/kg of feed (Smith et al., 2000; Smith, 2002). These earlier studies indicated the likely presence of a compound or

compounds in the Gungurru kernel meal that had a negative effect on shrimp growth rate. The results of the current study suggest that the compounds present in the cultivar Gungurru that adversely affected performance are not present in the new cultivars of *L. angustifolius*.

The iso-nitrogenous replacement of fishmeal with solvent-extracted soybean meal and with two lupin kernel meals, at a moderate and a high inclusion levels in the shrimp feeds, has demonstrated that the new cultivars of *L. angustifolius* perform equally to solvent extracted soybean meal. Furthermore, even at the high level of inclusion, in which more than 40% of the dietary protein was from the soybean meal or lupin kernel meal, the growth response did not differ from, or was better than, that of the fishmeal-based basal diet. Sudaryono et al. (1999b) replaced solvent extracted soybean meal that was included at 300 g/kg of feed, with *L. albus* kernel meal in diets for *P. monodon.* They found a progressive decrease in growth rate with increasing replacement. Their results clearly demonstrated the inferiority of *L. albus* in comparison to solvent extracted soybean meal. In a separate study with juvenile *P. monodon*, Sudaryono et al. (1999c) using a diet containing 300 g/kg of "defatted" soybean meal as a control, compared the response of the shrimp to diets in which the soybean meal had been replaced on an iso-nitrogenous basis with various lupin products, including kernel meal from *L. angustifolius.* However, they did not report the cultivar of *L. angustifolius* that they used in this study. Since the study was carried out before 1999, there is a reasonable probability that the cultivar was Gungurru. Their results showed no difference between the growth rates of the shrimp fed the soybean-based control diet and that containing *L. angustifolius* kernel meal at an inclusion level of 360 g/kg. Though these results are consistent with the results of the current study, they appear to be in contrast to the results of Smith et al. (2000) and Smith (2002), in that with the cultivar Gungurru at this inclusion level, a decrease in performance would be expected relative to that of the soybean meal based diet. This inconsistency may be due to a cultivar other than Gungurru being used in the Sudaryono et al. (1999c) study. Alternatively, this may have been a chance result as there were only three replicate tanks, each containing five shrimp, assigned to each treatment in the study.

The ingredient composition of diets in Experiment 1 differed from those in Experiment 2 and 3 due to non-availability of some of the ingredients. At the same time, the formulated levels for protein and lipid, on 'as used' basis, were reduced from 410 g/kg and 100 g/kg, respectively, to levels that were more widely used in commercial feeds for *P. monodon* (380 g/kg for protein and 80 g/kg for total lipid). The protein content of the kernel meals from Katanning used in Experiment 1 and 3, were more representative of typical commercial lupin production than the samples obtained from Wongan Hills (mean protein content of 427 g/kg DM compared with 478 g/kg DM, respectively). Furthermore, the protein content of particular cultivars from Wongan Hills was higher than that of the same cultivar grown at Katanning. However, within each experiment the lupin kernel meal was included in the feeds on an iso-nitrogenous basis, replacing an equal amount of fishmeal protein. Across the experiments they were included to provide the same proportion of the dietary crude protein (41.5% ± 0.82%). The performance of the cultivars grown at Katanning was consistent with the typical commercial sample obtained from Commercial Bulk Handling (CBH Mixed). These two groups of kernel meals provide a useful comparison between products that are commercially available and products with greater protein content which are possibly more useful to feed manufacturers. Comparisons between Experiment 1, and Experiments 2 and 3 are not straight forward, as the basal diet formulation differed. However, in Experiment 1, Kalya from Katanning performed as well as the basal diet and the other kernel meals. In Experiment 3, the Kalya from Katanning at a similar, high inclusion level performed as well as the basal diet, and its performance can be compared directly

with the performance of the cultivars from Wongan Hills (Experiment 2). This comparison indicates that the higher protein kernel meals from Wongan Hills did not perform any better than the more typical products from Katanning when used on an iso-nitrogenous basis.

The amino acid composition of lupin protein is similar to that of soybean but is characterized by relatively high levels of arginine, ~11 g/16 g N, which is about 40% greater the level in soybean protein (6.8 g /16 g N) (Table 24.2). However, lupin protein has relatively low levels of methionine, ~ 0.8 g/16 g N, or about half that of soybean protein. Hence, the total sulphur amino acid content (methionine+cysteine) is also low, ~ 2.4 g/16 g N. In shrimp feeds with a crude protein content of 38% 'as used', the recommended amount of methionine is 9.1 g/kg (Akiyama *et a*l. 1991). This is exceeded in the basal diet that was used in Experiment 3, which contains 10.3 g/kg of methionine (Table 26.8). The replacement of fishmeal with both soybean meal and the lupin kernel meals resulted in a decrease in the methionine content of the diets below the recommended content, particularly at the higher inclusion levels of these plant protein sources (7.3 g/kg and ~ 5.0 g/kg, respectively)(Table 26.8). These diets had very similar gross nutrient composition, so one might expect that the response of the shrimp would be sensitive to the methionine content, especially if it became limiting with the replacement of fishmeal with the soybean or lupin kernel meal. However, there was no difference in the growth response of the shrimp. These results indicate that the reported requirements of juvenile *P. monodon* for methionine (Akiyama et al. 1991; Millamena et al., 1996) are an overestimate of the minimum dietary specifications for methionine or methionine+cysteine. It appears that commercial feeds with 380 g/kg crude protein could be formulated with minimum specifications for methionine and methionine+cysteine of at least 6.0 g/kg, and 10.6 g/kg, respectively. It also indicates that with a dietary protein content of 380 g/kg, optimal growth rates can be achieved even when the amino acid profile of the diet does not closely match that of the carcass or muscle of the shrimp. This appears to conflict with widely held paradigm and warrants further examination

In conclusion, this study has demonstrated that lupin kernel meal can be used to replace at least 40 % of the fishmeal protein in diets for *P. monodon.* It has also shown the similarity in performance of the new cultivars of *L. angustifolius* that represent about 80% of Australia's current production. It appears that these cultivars can be used at higher inclusion levels than the older cultivar, Gungurru, without having an adverse affect on the growth of *P. monodon.* The study has also demonstrated that the new cultivars of *L. angustifolius* perform equally to solvent extracted soybean meal when used on a protein-equivalent basis and that the higher protein kernel meals from Wongan Hills did not perform any better than the more typical products from Katanning. From the amino acid analysis of the diets used in the experiments, it appears that that the reported requirements of juvenile *P. monodon* for methionine are overestimates. Further clarification of this issue is warranted as it is likely that formulators are restricting the inclusion level of lupins in shrimp feeds in order that they meet this over-specification for methionine.

26.5 References

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Table 26.1 Proximate composition (g/kg DM, unless otherwise stated) of kernel meals from cultivars of *L. angustifolius* and solvent extracted soybean meal that were evaluated in growth response experiments.

Ingredients[1]	Moisture (as used)	Ash	Crude protein	Total lipid	NFE	Energy (MJ/kg DM)
Experiment 1						
Kalya (KT)	101	36	418	96	451	20.7
Mandelup KT)	101	30	416	94	456	20.7
Tanjil (KT)	101	30	413	105	449	21.1
Belara (KT)	101	30	407	103	422	n.d.
Walan 2173(KT)	103	31	458	94	412	20.9
Myallie (KT)	102	31	453	89	422	21.8
Experiment 2						
Kalya (WH)	71	37	494	80	389	20.6
Mandelup (WH)	69	34	468	81	416	20.6
Tanjil (WH)	63	34	480	88	398	20.6
Wonga (WH)	66	33	470	88	409	20.6
Myallie (KT)	102	31	453	89	422	21.8
Experiment 3						
Soybean meal	103	72	551	43	334	20.3
Kalya (KT)	101	36	418	96	451	20.7
CBH Mixed	102	28	426	90	456	n.d.

[1] The region in Western Australia where the lupins were grown is indicated in parentheses after the cultivar name: KT = Katanning, WH = Wongan Hills.

Table 26.2 Amino acid composition (g /16 g N) of fishmeal, soybean meal and lupin kernel meals used in Experiment 3.

Amino acid	Fishmeal (Chilean)	Soybean (Solvent)	Kalya (KT)	CBH Mixed
Alanine	7.0	4.6	3.6	3.4
Arginine	5.6	6.8	10.6	11.7
Aspartic acid	10.3	12.6	11.6	11.2
Cysteine*	1.3	1.7	1.7	1.6
Glutamic acid	14.5	19.8	23.8	22.6
Glycine	6.0	4.2	4.3	4.1
Histidine	2.7	2.3	2.3	2.1
Isoleucine	4.8	4.4	3.9	3.9
Leucine	8.6	7.8	7.1	7.2
Lysine	7.3	6.2	3.2	4.3
Methionine	3.7	1.7	0.9	0.6
Phenylalanine	4.3	5.2	4.0	3.9
Proline	5.5	4.4	6.0	6.6
Serine	4.5	5.7	5.6	5.2
Threonine	5.3	4.7	4.2	3.7
Tyrosine	3.4	3.4	3.7	4.0
Valine	4.7	4.4	3.4	3.7
Methionine+cysteine	5.0	3.4	2.5	2.3

* Determined as cysteic acid derived from conversion of each molecule of cysteine to one molecule of cysteic acid, and each molecule of cystine to two molecules of cysteic acid.

Table 26.3 Experiment 1. Ingredient composition (g/kg, as used) of feeds used to examine the response of black tiger shrimp to inclusion of lupin kernel meals from Katanning.

Ingredient (g/kg as used)	Basal	Kalya (KT)	Mandelup (KT)	Tanjil (KT)	Belara (KT)	Walan 2173	Myallie (KT)
Fishmeal, Prime Peruvian	356	140	140	140	140	140	140
Langoustine meal	100	100	100	100	100	100	100
Squid meal	50	50	50	50	50	50	50
Gluten (wheat)	60	60	60	60	60	60	60
Lupin kernel meal [1]	–	501	517	506	523	450	450
Flour (wheat)	359	90	75	91	74	138	136
Lecithin (soybean)	10	10	10	10	10	10	10
Soybean oil	22	–	–	–	–	–	–
Cod liver oil	10	14	14	8	8	18	20
Binder (Aquabind)	30	30	30	30	30	30	30
Cholesterol	0.2	0.8	0.8	0.8	0.8	0.8	0.8
Other Ingredients [2]	3.0	3.0	3.0	3.0	3.0	3.0	3.0

[1] Feed identifier at the head of the column indicates which lupin cultivar was used

[2] Other ingredients includes (g/kg): Vitamin premix, 2.0; Vitamin C (Stay C), 1.0; Ethoxyquin (Banox E), 0.2; Astaxanthin (Carophyll Pink), 0.5;

Table 26.4 Experiment 2. Ingredient composition (g/kg, as used) of feeds used to examine the response of black tiger shrimp to inclusion of lupin kernel meals from Wongan Hills.

Ingredient (g/kg as used)	Basal	Kalya (WH)	Mandelup (WH)	Tanjil (WH)	Wonga (WH)	Myallie (KT)
Fishmeal, Prime Peruvian	389	150	150	150	150	150
Krill meal	100	100	100	100	100	100
Gluten (wheat)	50	50	50	50	50	50
Lupin kernel meal [1]	–	351	355	358	368	396
Starch (wheat)	210	100	97	97	88	66
Flour (wheat)	200	200	200	200	200	200
Lecithin (soybean)	10	–	–	–	–	–
Mixed vegetable oil	7	–	–	–	–	–
Cod liver oil	–	14	14	11	10	3
Binder (Aquabind)	30	30	30	30	30	30
Cholesterol	1	1	1	1	1	1
Other ingredients [2]	4	4	4	4	4	4

[1] Feed identifier at the head of the column indicates which lupin cultivar was used andi its source. WH = Wongan Hills; KT = Katanning

[2] Other ingredients includes (g/kg): Vitamin premix, 2.0; Vitamin C (Stay C), 1.0; Ethoxyquin (Banox E), 0.2; Astaxanthin (Carophyll Pink), 0.5;

Table 26.5 Experiment 3. Ingredient composition (g/kg, as used) of feeds used to compare the response of black tiger shrimp to the iso-nitrogenous replacement of fishmeal with soybean meal (solvent extracted) and lupin kernel meals at two inclusion levels. M = Moderate inclusion level; H = High inclusion level.

Ingredient (g/kg as used)	Basal	Soybean (M)	Soybean (H)	Kalya (KT) (M)	Kalya (KT) (H)	CBH Mixed (M)	CBH Mixed (H)
Fishmeal, Prime Peruvian	389	250	150	250	150	250	150
Krill meal	100	100	100	100	100	100	100
Gluten (wheat)	50	50	50	50	50	50	50
Soybean meal (solvent ext'd)	–	191	330	–	–	–	–
Lupin kernel meal [1]	–	–	–	249	428	230	396
Starch (wheat)	210	152	111	111	37	128	66
Flour (wheat)	200	200	200	200	200	200	200
Lecithin (soybean)	10	10	10	5	–	–	–
Mixed vegetable oil	7	–	–	–	–	–	–
Cod liver oil	–	12	15	1	–	8	3
Binder (Aquabind)	30	30	30	30	30	30	30
Cholesterol	1	1	1	1	1	1	1
Other ingredients [2]	4	4	4	4	4	4	4

[1] Feed identifier at the head of the column indicates which product was used. KT = Katanning.

[2] Other ingredients includes (g/kg): Vitamin premix, 2.0; Vitamin C (Stay C), 1.0; Ethoxyquin (Banox E), 0.2; Astaxanthin (Carophyll Pink), 0.5;

Table 26.6 Description and source of ingredients used in feeds prepared for the growth response experiments. Unless otherwise stated, ingredients were obtained from sources in Australia.

Ingredient	Source
Fishmeal, Prime Peruvian, 68% CP.	Supplied by Ridley AquaFeeds, Narangba, Qld.
Langoustine meal.	Inual, Santiago, Chile. Supplied by Ridley AquaFeeds, Narangba, Qld.
Krill meal. Dried whole Antarctic krill, *Euphausia* spp	Inual-Tepual Ltd, Santiago, Chile.
Squid meal.	Japan. Supplied by Ridley AquaFeeds, Narangba, Qld.
Gluten (wheat). 76% CP	Janbak Industries Pty Ltd, Brisbane, Qld.
Flour (wheat)	White Wings, Brisbane, Qld
Starch (wheat)	Janbak Industries Pty Ltd, Brisbane, Qld
Lecithin (soybean). 76% lipid.	Janbak Industries Pty Ltd, Brisbane, Qld
Mixed vegetable oil.	Crisco. Goodman Fielder Consumer Foods, Macquarie Park, NSW
Cod liver oil.	Melrose Laboratories, Box Hill, Victoria
Cholesterol. 100%	ICN Nutritional Biochemicals, Cleveland, OH, USA
Binder (Aquabind)	Supplied by Ridley AquaFeeds, Narangba, Qld.
Astaxanthin (Carophyll Pink)	DSM Nutritional Products Australia P/L, Sydney, NSW
Vitamin Premix. Based on Conklin, 1997	Supplied by Rabar Pty Ltd, Beaudesert, Qld
Ascorbyl-2-polyphosphate (Stay-C)	DSM Nutritional Products Australia P/L, Sydney, NSW
Ethoxyquin (Banox E)	Adisseo Australia, Carole Park, Qld.

Table 26.7 Proximate composition (g/kg DM) of feeds used in the growth response experiments. Feeds are identified either as a Basal feed or by the lupin or soybean meal in the formulation and, where applicable, the inclusion level – medium (M) or high (H).

Feed[1]	Ash	Crude protein	Total lipid	NFE	Energy (MJ/kg DM)
Experiment 1					
Basal 1	108	437	114	341	20.8
Kalya (KT)	88	453	109	350	21.1
Mandelup (KT)	87	456	109	348	21.1
Tanjil (KT)	87	454	111	348	21.0
Belara (KT)	87	454	105	354	21.0
Walan 2173(KT)	87	456	110	347	21.0
Myallie (KT)	86	452	106	356	21.0
Experiment 2					
Basal 2	89	426	88	397	20.7
Kalya (WH)	60	419	87	434	20.9
Mandelup (WH)	59	414	110	417	21.0
Tanjil (WH)	59	416	86	439	21.0
Wonga (WH)	60	424	87	429	21.0
Myallie (KT)	61	423	81	435	21.0
Experiment 3					
Basal 2	89	426	88	397	20.7
Soybean meal (M)	80	421	97	402	20.7
Soybean meal (H)	72	420	100	408	20.8
Kalya (KT) (M)	73	423	99	405	20.7
Kalya (KT) (H)	61	417	100	422	20.9
CBH Mixed (M)	72	417	102	409	20.8
CBH Mixed (H)	59	413	109	419	21.0

[1] The region in Western Australia where the lupins were grown is indicated in parentheses after the cultivar name: KT = Katanning, WH = Wongan Hills.

Table 26.8 Amino acid composition of basal diet and diets with the high inclusion level of soybean meal and lupin kernel meal used in Experiment 3 (g/kg DM).

Amino acid	Basal 2	Soybean (H)	Kalya (KT) (H)	CBH Mixed (H)
Alanine	19.9	17.0	15.9	15.9
Arginine	26.2	26.2	30.5	31.3
Aspartic acid	30.7	34.0	33.0	33.3
Cysteine*	4.4	4.6	4.6	4.3
Glutamic acid	62.3	70.2	77.8	76.6
Glycine	19.6	16.6	16.3	16.4
Histidine	10.4	9.0	9.0	9.2
Isoleucine	14.9	14.5	13.6	14.3
Leucine	25.9	25.8	24.5	24.6
Lysine	24.2	21.5	19.7	19.8
Methionine	10.3	7.3	6.0	5.8
Phenylalanine	15.0	16.0	14.2	14.4
Proline	18.1	19.1	19.0	19.1
Serine	14.5	16.6	16.2	15.7
Threonine	14.5	13.8	12.8	12.9
Tyrosine	10.7	11.0	10.9	11.0
Valine	16.8	15.6	14.3	14.9
Methionine+cysteine	14.7	11.9	10.6	10.1
Total AA (g/kg)	338	339	338	339
Diet CP (g/kg)	426	420	417	413
Total AA/Diet CP (%)	79	81	81	82

* Determined as cysteic acid from conversion of each molecule of cysteine to one molecule of cysteic acid and each molecule of cystine to two molecules of cysteic acid.

Table 26.9 Experiment 1: Growth response parameters of shrimp fed for 50 d with feeds containing lupin kernel meals from Katanning (KT)*. Feeds are identified either as the Reference, the Basal or by the lupin meal in the formulation. s.e.m. = standard error of the mean.

Feed[1]	Initial weight (g)	Growth rate (g/wk)	DGC (%/d)	Feed allocation (g/tank)	FCR	Survival (%)
Reference diet	6.93	1.29[a]	1.24[a]	155[bc]	3.5[a]	100
Basal 1	6.82	0.80[bc]	0.83[bc]	126[d]	4.6[ab]	93
Kalya (KT)	7.01	0.92[b]	0.95[b]	195[a]	5.9[bc]	93
Mandelup (KT)	7.05	0.87[bc]	0.89[bc]	179[ab]	5.8[bc]	93
Tanjil (KT)	6.89	0.72[c]	0.77[c]	166[bc]	7.1[c]	93
Belara (KT)	6.85	0.78[bc]	0.83[bc]	164[bc]	6.0[bc]	93
Walan 2173(KT)	6.98	0.91[bc]	0.93[bc]	162[bc]	5.0[b]	90
Myallie (KT)	6.94	0.73[bc]	0.78[bc]	150[cd]	5.8[bc]	100
s.e.m.	0.065	0.068	0.058	9.6	0.49	3.7

* Means within a column having the same superscript letter are not significantly different ($P > 0.05$).

Table 26.10 Experiment 2: Growth response parameters of shrimp fed for 50 d with feeds containing lupin kernel meals from Wongan Hills (WH) or Katanning (KT)*. Feeds are identified either as the Reference, the Basal, or by the lupin meal in the formulation. s.e.m. = standard error of the mean.

Feed[1]	Initial weight (g)	Growth rate (g/wk)	DGC (%/d)	Feed allocation (g/tank)	FCR	Survival (%)
Reference	3.19	1.18[a]	1.57[a]	110[a]	2.7	100
Basal 2	3.34	0.70[b]	1.05[b]	86[b]	4.0	90
Kalya (WH)	3.33	0.74[b]	1.11[b]	82[b]	3.1	87
Mandelup (WH)	3.33	0.75[b]	1.12[b]	86[b]	3.2	90
Tanjil (WH)	3.31	0.72[b]	1.09[b]	81[b]	3.2	83
Wonga (WH)	3.27	0.80[b]	1.19[b]	88[b]	3.1	80
Myallie (KT)	3.32	0.79[b]	1.18[b]	85[b]	3.0	90
s.e.m.	0.041	0.060	0.067	3.2	0.40	5.7

* Means within a column having the same superscript letter are not significantly different ($P > 0.05$).

Table 26.11 Experiment 3: Growth response parameters of shrimp fed for 50 d with feeds containing either soybean meal or lupin kernel meals*. Feeds are identified either as the Reference, the Basal or by the lupin or soybean meal in the formulation and its inclusion level – medium (M) or high (H).

Feed	Initial weight (g)	Growth rate (g/wk)	DGC (%/d)	Feed allocation (g/tank)	FCR	Survival [a] (%)
Reference	3.19	1.18[a]	1.57[a]	110[a]	2.7[ab]	100[a]
Basal 2	3.32	0.70[b]	1.05[c]	86[de]	4.0[c]	90[abc]
Soybean meal (M)	3.31	0.81[b]	1.19[bc]	73[f]	2.5[a]	80[c]
Soybean meal (H)	3.41	0.78[b]	1.14[bc]	103[ab]	3.8[bc]	90[abc]
Kalya (KT) (M)	3.33	0.86[b]	1.25[b]	88[cde]	2.9[bc]	93[ab]
Kalya (KT) (H)	3.31	0.77[b]	1.15[bc]	97[bc]	3.5[abc]	97[a]
CBH Mixed (M)	3.37	0.74[b]	1.11[bc]	79[ef]	3.1[abc]	77[c]
CBH Mixed (H)	3.36	0.81[b]	1.18[bc]	96[bcd]	3.3[abc]	83[bc]
s.e.m.	0.044	0.063	0.067	3.8	0.40	5.4

* Means within a column having the same superscript letter are not significantly different ($P > 0.05$).

[a] Significance of differences are from ANOVA of the arcsine transformed data. For the purpose of clarity the untransformed data is presented.

27.0 Response of the black tiger prawn, *Penaeus monodon* to feed containing the lupin alkaloid, gramine[a]

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Abstract

In this study we have examined the effect of the lupin alkaloid, gramine, when included in a feed for the black tiger prawn, *Penaeus monodon*. Alkaloids are generally classified as anti-nutritional factors that can limit the use of legumes in aquaculture feeds. Gramine is the predominant alkaloid in the Yellow lupin (*Lupinus luteus*), but is present at very low levels in the Australian cultivar Wodjil. Therefore, Wodjil is more susceptible to aphid damage, and so plant breeders are interested in increasing its gramine content to provide better protection for the crop. The rate of leaching loss of gramine from feeds was determined, and dose-response studies was carried with juvenile *P. monodon* to determine the effect of dietary gramine content on feeding behaviour, feed intake, growth rate, survival and digestive gland histology. Gramine leached from the feeds quite rapidly with about 20% of the gramine lost in the first hour. High levels of gramine significantly reduced feed intake in the first 15 min after distribution of the feed. However, thereafter over the 6 h that were closely monitored, feed intake did not appear to be different across treatments. The daily feed intake, growth rate and survival of the prawns was not affected by the concentration of gramine in the feed over the range of concentrations examined (0 to 902 mg/kg of feed, as used). In addition, no histological changes in the digestive gland of the prawns in response to the gramine content of the feed were observed. It is highly unlikely that commercial feeds using a 30% inclusion of Australian-produced lupin kernel meals would exceed the maximum level tested in this study. These data indicate that there is significant scope for plant breeders to increase the gramine levels in the Yellow lupin, cv. Wodjil from its current very low level to levels that will provide much better protection against aphids, without compromising the nutritional value of the kernel meal.

27.1 Introduction

Much of the recent increase in global aquaculture production has been brought about through the adoption of intensive farming practices using formulated feeds. Feeds used in the culture of carnivorous fish and crustaceans generally contain a high concentration of protein, much of which is presently obtained through the inclusion of fishmeal at between 200 and 300 g kg^{-1} of feed. In 2001, the feeding of these species required an estimated 16.7 million tonnes of aquafeeds, containing about 2.6 million tonnes of fishmeal (or 43.1% of the total global production) (FIN, 2004). However, world fishmeal production has remained relatively static at 6.2 million tonnes (IFFO, 2006) and is unlikely to increase further. Fishmeal production is also subject to sharp, periodic declines such as in 1998 when only 4.75 million tonnes were produced (Barlow, 2002). It is evident from these statistics that continued expansion of aquaculture will

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not be possible if fishmeal is relied upon as the main source of protein in aquafeeds. Moreover, demand for fishery product from other sectors such as the pig, poultry and pet food industries will force fishmeal prices up until its usage in aquafeeds becomes uneconomical. In any event, if aquaculture is to become a net and increasing contributor to human food supplies, it is critical that aquafeeds become less reliant on fishmeal.

There has been a considerable amount of research evaluating alternative, terrestrial protein sources for use in aquaculture feeds (Lim et al., 2007). Much of the research interest has been directed towards the use of soybean meal, but more recent studies have extended to the use of field peas, canola and lupins. The nutritional value of a number of species and cultivars of lupins has been assessed for a wide variety of fish and prawn species (reviewed by Glencross, 2001). Rainbow trout *(Oncorhynchus mykiss)* have been used extensively as a test species, though there is an increasing body of work with Atlantic salmon (*Salmo salar*). In addition, there have been studies with seabreams, silver perch, Asian sea bass, carps, tilapia, milkfish and turbot, and with marine shrimp and freshwater crayfish (Glencross, 2001).

Lupin production in Australia is dominated by a number of cultivars of the narrow leafed lupin (= Australian sweet lupin, *Lupinus angustifolius*), which is recognised as having low levels of anti-nutritional factors. These include protease inhibitors, glucosinolates, saponins, tannins and alkaloids, though they contain appreciable amounts of oligosaccharides (Francis et al., 2001; Petterson et al., 1997). Though there is currently only limited production of Yellow lupins (*L. luteus*) in Australia, this species is seen as having great potential as an ingredient for the aquaculture feed industry as the kernel meal has significantly higher protein content than that of current cultivars of *L. angustifolius* and also has low levels of anti-nutritional factors (Petterson et al., 1997; Evans, 1998; Glencross et al., 2006). In particular, the current cultivar of *L. luteus* grown in Australia, Wodjil, contains very low levels of alkaloids compared with the predominant European cultivar of *L. luteus*, Teo (32 mg/kg DM cv. 4087 mg/kg DM; Glencross et al. 2006).

Though alkaloids are found in most legume species, they have been found in high concentrations in wild or undomesticated stocks of lupins, occurring in both the phloem and the seeds. Alkaloids are a diverse group of nitrogen-containing compounds that are toxic to many organisms and include compounds such as nicotine, quinine and cocaine. They are produced by plants as chemical defence agents against pests (Petterson et al., 1991). The most obvious action of the alkaloids is they deter insects and animals from feeding on the plants or seeds, possibly through the bitter taste that they impart (Ridsdill-Smith et al., 2004; Urbańnska et al. 2006). As *L. luteus* cv. Wodjil contains very low levels of alkaloids, it is prone to infestation by aphids, with a consequential decrease in crop yield and the need for regular chemical spraying to combat the aphids. Plant breeders are interested in gaining information about the response of key aquaculture species to different concentrations of alkaloids in the feed, to determine the scope that they have to increase the alkaloid content of Wodjil, without having a deleterious effect on its nutritional value.

Alkaloids have been shown to affect feed intake when included in feeds for Rainbow trout, *O. mykiss* (de la Higuera et al., 1988). Gramine is the predominant alkaloid in *L. luteus* and at a threshold concentration of between 100 mg/kg and 500 mg/kg, has a significant effect on the feed intake of *O. mykiss* (Glencross et al. 2006). However, there appears to be no information in the literature about its effect on prawns. In this study, we have measured the rate that gramine leaches from feed pellets when they are placed in seawater and have carried out a dose-response experiment with a range of inclusion levels of gramine in the feed of juvenile *Penaeus monodon*

(0 to 902 mg/kg of feed). In the does-response experiment we have examined the effect of gramine on feeding behaviour, feed intake, growth, survival and digestive gland histology.

27.2 Materials and methods

27.2.1 Ingredients and diet preparation

Purified gramine (Aldrich, Cat. #10806, Sigma Aldrich, Castle Hill, NSW, Australia) was dissolved in methanol and thoroughly mixed with α-cellulose (Sigma Aldrich) to form a slurry. The solvent was evaporated from the slurry in a rotary evaporator to form a free-flowing powder, which was dried further in a desiccator under vacuum (Glencross et al., 2006). The α-cellulose was used as a carrier for the gramine to facilitate its dispersion and homogeneous distribution through the mixed feed ingredients. The gramine/cellulose (nominally 10 g/kg DM) was added to formulations at the expense of gramine-free α-cellulose in the basal feed (0 g/kg gramine) to make series of ten diets in which the gramine content varied between 0 to 902 mg/kg (0, 42, 79, 112, 201, 272, 413, 686, 827, 902 mg/kg) (Table 27.1). The proportions of gramine/cellulose and cellulose were the only changes to the formulations of the series of feeds. Two additional feeds were prepared. The first contained 300 g/kg of low-alkaloid lupin kernel meal from the Yellow lupin, *L. luteus* cv. Wodjil (Table 27.1), while the second was essentially of the same formulation but which contained 300 g/kg of a kernel meal with high levels of gramine from *L. luteus* cv Teo (Glencross et al., 2006). The feeds were nutritionally balanced and contained 425 g/kg crude protein and 68 g/kg crude fat, on a dry matter basis.

Before being weighed out, dry ingredients were ground to ensure all of the material passed through a 710 µm screen. Each diet was prepared individually rather than using a bulk, pre-mixed base, to avoid the possibility of an unrepresentative sample of mixture being used for any particular feed. The weighed ingredients were thoroughly combined in a planetary mixer before a volume of water equivalent to approximately 40% of the dry weight of ingredients was added, and mixed further to form a crumbly dough. The dough was extruded through the meat grinder attachment of a Hobart mixer (Hobart Corporation, Troy, OH, USA). The extruded, spaghetti-like strands (~3 mm diameter) were steamed for 5 min in an atmospheric steamer (Curtin & Son, Sydney, Australia), air-dried overnight in a forced-draught cabinet at 40°C and broken into pellets 5 to 8 mm long. The pellets were stored at -20°C until used.

27.2.2 Leaching experiment

The leaching rate of gramine from three representative feeds (112 g/kg, 413 mg/kg and 902 mg/kg) was determined in a time-series experiment. Also included in the experiment was the feed containing 300 g/kg of Teo, which contained high levels of naturally occurring gramine. Four samples of each of the four feeds were weighed and placed in labelled beakers containing 300 mL of seawater at room temperature (22°C). A fifth sample was weighed out and retained as an untreated sample (0 h). The beakers were gently agitated on an orbital shaker at 60 rpm. After 0.5 h, 1 h, 2 h, and 4 h the feed pellets from one beaker for each treatment, were removed from the water, rinsed briefly in distilled water, dried at 40°C and analysed for gramine content. The gramine (mg) remaining in the feed pellets was expressed as a percentage of the amount of gramine (mg) in the feed pellets when they were placed in the beaker. The initial amount of gramine was calculated using the initial weight of feed pellets and the concentration of gramine determined from the analysis of the 0 h sample. A correction factor was also applied to adjust for dry matter loss over the time that the pellets were in the water.

27.2.3 Feeding behavioural response

An experiment was carried out to determine the extent to which the feeding behaviour of juvenile black tiger prawns, *P. monodon,* was altered in response to the concentration of gramine in the feed. It was necessary to establish if the prawns were delaying consumption of the feed until a significant proportion of the gramine had leached from it. The basal feed and the three gramine feeds (112 mg/kg, 413 mg/kg and 902 mg/kg gramine) that were used in the leaching experiment were used in this experiment. Five prawns of between 4.0 g and 5.0 g were placed in each of 28 circular, polyethylene tanks of 100 L capacity (0.6m diameter, 0.35 m depth). Hence, this array of tanks provided seven replicate tanks for each treatment. The tanks were supplied with filtered (20 µm) and heated seawater, flowing at 0.5 L/min, maintaining the tank temperature at 29 ± 0.2°C (maximum range 28.2°C to 29.6°C). The light cycle in the aquarium room was adjusted so that the lights came on at 1400 h. and were turned off at 0430 h leaving the room in darkness.

Initially, to establish the base line variability in the feeding behaviour of the prawns, all tanks were fed the basal diet for 7 days and the feeding patterns recorded on the last 5 days. Thereafter, the prawns were fed their allocated diets for two weeks, with their feeding patterns observed on Mondays through to Fridays. The prawns were fed twice daily at 0900 h and 1630 h. All uneaten food and faeces was removed by siphoning at 0800 h under red light. Starting at 0900 h, each tank of prawns was fed at 30 second intervals with a know number and weight of feed pellets. The number of pellets that remained uneaten was counted *in situ* and recorded under low intensity red light 15 min, 30 min, 1 h, 2 h, 3 h, 4 h and 6 h after feeding. The weight of feed consumed within each time period was estimated from the number of feed pellets consumed and the average weight of the pellets. The data was examined in terms of actual amount of feed eaten in each of the time periods, and the cumulative amount of feed eaten over the 6 h. In addition, the weight of feed eaten in each of the time periods was examined as a percentage of the total amount of feed eaten in the 6 h.

27.2.4 Growth response experiment

In a 50 d growth experiment juvenile black tiger prawns were fed a series of ten diets in which the gramine content varied between 0 to 902 mg/kg (Table 27.1). Two additional treatments were included in the experiment: (a) the diet containing 300 g/kg of low-alkaloid lupin kernel meal from *L. luteus* cv. Wodjil (Table 27.1), and (b) a high-performing, high-protein, commercial prawn feed (Lucky Star, Hung Kuo Industrial Co, I-Lan, Taiwan) that was used as a reference diet. Four replicate tanks were assigned to each of the ten-gramine series of feeds, except for the 0 g/kg gramine feed for which there were 6 replicates. There were also 6 replicate tanks assigned to the Wodjil feed and to the Lucky Star feed. The dietary treatments were assigned to tanks in a completely randomised design.

Juvenile black tiger prawns were obtained for the experiment from a commercial prawn farm in southeast Queensland, Australia. The prawns were of a narrow size range (2.5 to 3.0 g) and were distributed in an array of 54 tanks with 6 prawns in each tank. The circular, polyethylene tanks were of the same design as used in the feeding behaviour experiment. The tanks were also provided with filtered (20 µm) and heated seawater, flowing at 0.5 L/min, maintaining the tank temperature at 29 ± 0.2°C (maximum range 28.2°C to 29.6°C). The aquarium room was illuminated on a 12 h light and 12 h dark cycle. The prawns were maintained in the tanks and fed the basal feed (0 g/kg gramine) twice daily for 7 days prior to the start of the experiment.

At the start of the experiment, the prawns were weighed and redistributed within the tanks with

5 prawns per tank, so that there was closely matching biomass in each tank (mean ± SD, 16.1 ± 0.37 g), with the initial mean weight of the prawns 3.2 ± 0.24 g. Prawns were fed the assigned feeds twice daily, at 0830 and 1700 h, to slight excess. The amount of feed given to each tank was recorded, and adjusted daily according on the amount left uneaten in the previous 24 h. The prawns were individually weighed after 25 d and finally after 50 d. Any prawns that died during the experiment were replaced with a tagged prawn of similar size, to maintain stocking density. Though individual weights were recorded, only the mean weight of prawns within each tank was used in the data analysis. The tagged replacement prawns were not included in the growth response data.

At the conclusion of the experiment, representative samples of prawns were taken to identify and quantify any changes to the digestive gland that might be related to the gramine content of the prawns' diet. Five randomly selected prawns from each of the 10-gramine treatments were examined. To prepare the digestive gland for histology, the prawns were individually chilled in ice/seawater slurry; the cephalothorax was dissected from the abdomen and immediately cut it in half longitudinally (sagittal section) and placed in Davidson's fixative (Bell and Lightner, 1988). Tissues were fixed for 24 hours and then transferred to 70% ethanol for storage prior to routine tissue processing (Bell and Lightner, 1988). Tissue sections (5 µm) were stained with haematoxylin and eosin stain (Clinipure, HD Scientific, Wetheril Park, NSW, Australia). The digestive gland sections were examined using light microscopy (100 X and 200 X magnification). Images were captured using a Leica DC 200 camera and computer software. Comparisons were made between prawns fed the basal feed and prawns fed the gramine-containing feeds to determine variations in digestive gland tissue and cellular structure.

27.2.5 Chemical analysis

The dry matter content of the ingredients and feeds was determined by drying at 105°C for 16 h. The ash content was determined by heating a weighed dry sample at 550°C for 6 h (method 938.08, AOAC International 1999) and the crude protein (6.25 x total N) by a modified Kjeldahl digestion (Bradstreet, 1965) followed by colorimetric analysis using the indophenol colour reaction (Searle, 1984) in a Technicon segmented flow autoanalyser (Technicon Instruments Corporation, Tarrytown, NY, USA). Crude fat was determined gravimetrically following soxhlet extraction with petroleum ether (AOAC International, 1999).

Gramine content of the cellulose/gramine mixture and feeds was determined by extraction with trichloroacetic acid then extraction from the aqueous layer with methylene chloride, which was drawn off and made up to a known volume. The gramine concentration was determined by capillary gas chromatography using a non-polar column (HP-1, 30 m, Hewlett-Packard Company, PA, USA), and detected with a flame ionisation detector (Harris and Wilson, 1988). Quantification was obtained using known standards.

27.2.6 Statistical analysis

The feeding behaviour data was analysed using both ANOVA and regression analysis. The absolute feed intake data was analysed without transformation, while the percentage data was analysed un-transformed and following arcsine transformation. Though there were only four diets in the experiment, the response to gramine content of the diets was examined using regression analysis with a 2nd order polynomial model.

Data from the dose-response series of treatments in the feeding experiment were analysed using a linear regression analysis (REGN, Queensland Department of Primary Industries, Brisbane,

Australia). The analytically determined gramine content of the feeds (mg/kg DM) were used as the independent variable, with growth rate, feed allocation and arcsine transformed survival data as dependent variables. The response of the prawns fed the 0 g/kg gramine diet, the Yellow lupin diet and the Reference diet were also analysed using an Analysis of Variance. The Lucky Star feed was used primarily to assess the performance and quality of the prawns. Differences between treatment effects were tested for significance with a *t*-test, only when the '*F*' test of the ANOVA was significant ($P < 0.05$) (Fischer's protected *t*-test, Snedecor and Cochran, 1989).

27.3 Results

When immersed in seawater, the gramine in the prepared feeds leached out of the pellets at a rate of about 20% /h (Figure 27.1). The leaching rate was the same with naturally-occurring gramine in the feed containing the kernel meal from *L. luteus* cv. Teo.

Our observations of the feeding behaviour of the prawns showed that after the feed was placed in the tanks, there was a high level of feeding activity in the initial 15 min followed by a lower level of activity that continued for the 6 hours over which observations were made (Figure 27.2). In the first 15 min, the feed intake with the basal diet was markedly greater than with the 903 mg/kg gramine feed (35 g/prawn cf. 18 mg/prawn, respectively). However, the standard error of these means was relatively large (± 5.4 mg/prawn) and the differences were not found to be statistically significant. When a quadratic regression was fitted to the data, there appeared to be a response of decreasing feed intake with increasing gramine content (Figure 27.3). When the feed intake in the first 15 min was expressed as a percentage of the feed eaten over the 6 hours, there was a significant difference between the feed intake of prawns fed the 903 mg/kg gramine feed (20%) and that with both the basal feed (38%) and the 112 mg/kg gramine feed (33%) (standard error ± 4.0%).

The amount of feed allocated to tanks of prawns over 50 d did not change with the level of gramine in the feed, $Y = 91.4 - 0.0036X$, $R^2 = 0.0265$ (Figure 27.4). Though survival (%) of the prawns appeared to decrease slightly with increasing gramine content in the feed, regression analysis of the arcsine transformed data showed that there was not a significant effect of gramine content ($Y = 65.17 - 0.0160X$, $R^2 = 0.0822$). The growth rate of the prawns did not appear to be affected by the amount of gramine in the feed when the feed was place in the tanks (Figure 27.5).

Histological examination of the digestive glands of representative samples of prawns from all treatments showed that there were no visual differences in tissue and cellular structure that could be associated with the dietary gramine content.

27.4 Discussion

The feed intake, growth rate and survival of the juvenile black tiger prawns were not affected by the concentration of gramine in the feed over the range examined (0 to 902 mg/kg of feed, as used). In addition, there did not appear to be any histological changes in the digestive gland of the prawns in response to the gramine in the feed. This contrasts with the results reported in rainbow trout (*O. mykiss*), in which feed intake and growth were significantly depressed by gramine levels of > 100 mg/kg (Glencross et al., 2006). It is possible that the difference in the response observed with trout and prawns may be influenced by the feeding behaviour of the species. While trout tend to feed as pellets are offered, prawns will initially feed to apparent satiation but then continue to feed at a lower rate of consumption for a prolonged period and

will consume feed that has been in the water for many hours.

The leaching rate of gramine from the feed was much higher than expected. Gramine is considered to be practically insoluble in water (The Merk Index, 1976) and so would not be expected to dissolve and leach from the feed pellets at an appreciable rate. A plausible reason for this high leaching rate has not been established. However, from the leaching experiment, it is also apparent that naturally occurring gramine in Yellow lupin cv. Teo, leached from the diets at a similar rate to that of the purified gramine. In the feeding behaviour study, the concentration of gramine in the feed appeared to reduce feed intake in the first 15 min after the feed had been distributed in the tanks (Figure 27.2), but thereafter there was no difference in the feed intake. However, though the cumulative feed intake was not significantly different among treatments, it appears that the shrimp fed the diets with high levels of gramine did not compensate over the 6 h for the initial setback in feed intake. However, from the feed intake data obtained in the growth response experiment, it appears that there was no significant difference in the daily feed intake across all treatments, suggesting that the shrimp may have gradually compensated through the day or night for the initial set-back in feed intake. This finding is supported by the data that shows there were no significant differences in growth rate among treatments. Since these diets were contained the same concentrations of nutrients, it is reasonable to accept that growth rate is strongly related to feed intake. These data suggests that when lupin kernel meals containing elevated levels of alkaloids are used in prawn feeds, consideration should be given to the fact that a significant proportion of the alkaloid material is likely to leach from the feed before all of it is consumed.

To put the amount of gramine in the feeds into perspective; at the highest inclusion level (902 g/kg), the initial amount of gramine in the feed was equivalent to that in a feed containing 30% lupin kernel meal with a gramine content of 3000 mg/kg. In comparison, the low-alkaloid cultivar Wodjil contains about 32 mg/kg DM of gramine and the high-alkaloid cultivar, Teo, contains 4087 mg/kg DM (Glencross et al., 2006).

While various cultivars of *L. angustifolius* have been used successfully in prawn feed formulations to replace fishmeal (Smith et al., 2007), *L. luteus* kernel meal has not been evaluated previously. *L. luteus* appears to have great potential as an aquafeed ingredient because it has higher protein content than current cultivars of *L. angustifolius*. The kernel meal of *L. luteus* has about 530 g/kg DM of crude protein whereas that of *L. angustifolius* generally has between 420 to 440 g/kg DM (Glencross, 2001). In this experiment the cultivar Wodjil was used to replace more than 60% of the fishmeal in the basal formulation without any effect on performance. This is a similar response to that observed with *L. angustifolius* and suggests that it would be a particularly useful ingredient for prawn feeds.

In conclusion, the inclusion of up to 900 mg kg^{-1} of gramine in the feed of black tiger prawns did not significantly affect the daily feed intake, growth response or survival of the prawns nor did it affect the histology of the digestive gland. It does not appear that the gramine had an adverse effect on the attractant qualities of the feed, as the greatest feed intake across all treatments occurred in the first 15 min after the feed had been distributed in the tanks. However, it does appear that that a relatively high concentration of gramine in the feed has an adverse effect on the palatability of the feed, though this only occurs in the first 15 min. Thereafter, possibly because of the relatively small amounts of feed being consumed and the rapid leaching of gramine from the feed, the initial gramine content of the feed had little affect on intake. These results suggest that there would not be an adverse effect on productivity if the prawns were fed with diets containing 30% of a lupin kernel meal that contained less than 3000 mg/

kg of gramine. Feed companies are unlikely to use more than 30% lupin kernel meal in prawn feeds and this gramine content is more than 150 times greater than that currently found in *L. luteus* cv Wodjil.

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Tables and Figures

Table 27.1 Ingredient composition (g/kg, as used) of key feeds used to examine the response of black tiger prawns to dietary gramine content. Only the gramine-containing feeds with the lowest and highest inclusion levels of gramine are shown. Formulated inclusion levels of gramine were: 50, 100, 150, 250, 350, 500, 700, 900, 1200 mg/kg.

Ingredient (g/kg as used)	Basal	G-50	G-1200	Wodjil
Fishmeal, Prime Peruvian	388.9	388.9	399.9	150.0
Krill meal	100.0	100.0	100.0	100.0
Gluten (wheat)	50.0	50.0	50.0	50.0
Cellulose	12.0	11.5	0.0	12.0
Gramine/Cellulose	0.0	0.5	12.0	0.0
Wodjil kernel meal	0.0	0.0	0.0	330.1
Lecithin (soybean)	10.0	10.0	10.0	10.0
Mixed vegetable oil	6.8	6.8	6.8	0.0
Cod liver oil	0.0	0.0	0.0	6.4
Wheat starch	197.5	197.5	197.5	106.8
Flour	200.0	200.0	200.0	200.0
Other s*	34.7	34.7	34.7	34.7

* includes (g/kg as used): Aquabind, 30; vitamin premix, 2; vitamin C (Stay C), 1; cholesterol, 1; carophyll pink, 0.5; Banox E, 0.2.

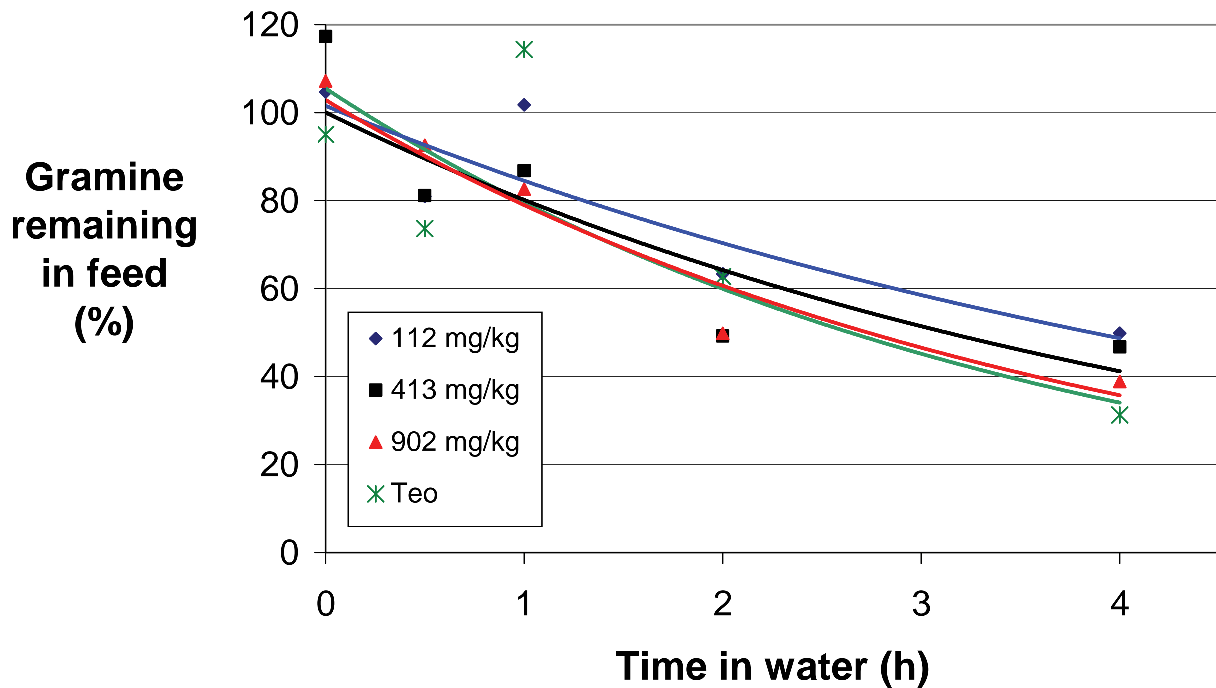

Figure 27.1 Decrease in gramine content of prepared feeds with time of immersion in seawater. Data standardised as a percentage of the initial amount of gramine in the feed.

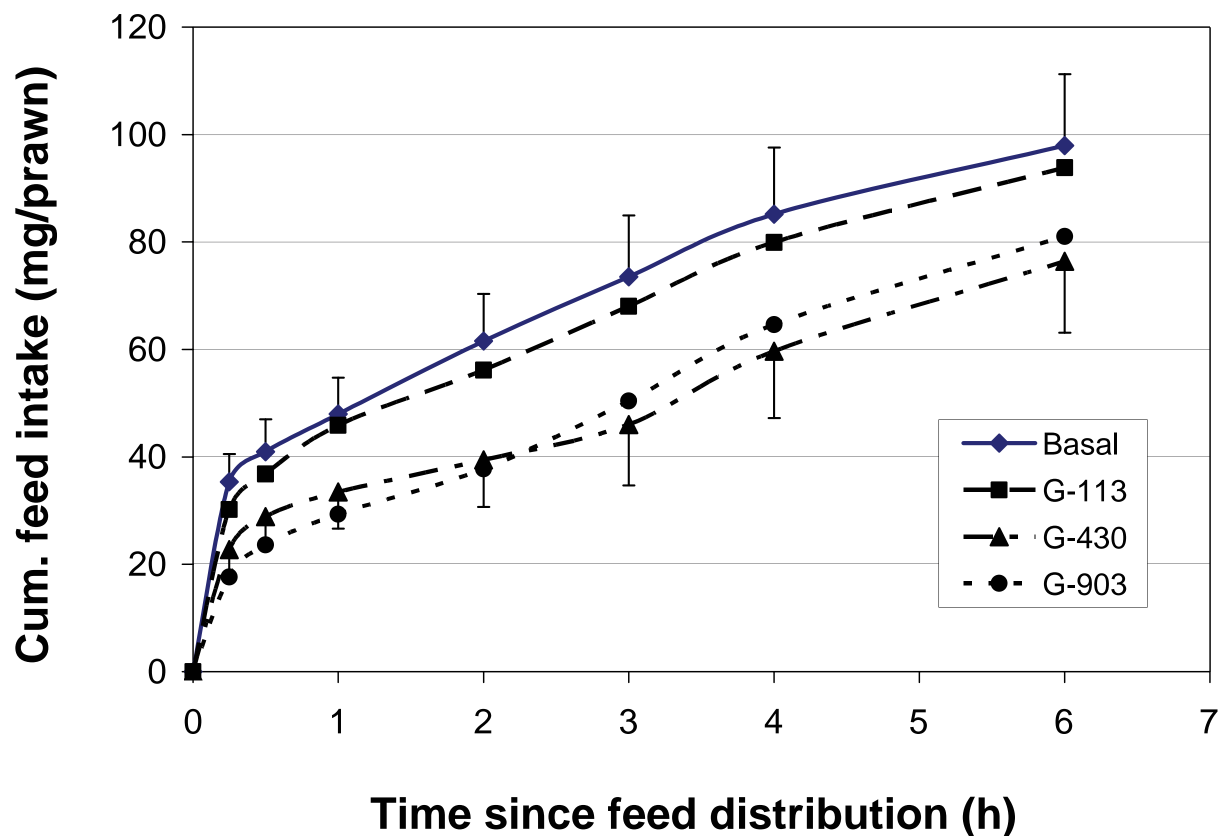

Figure 27.2 Average cumulative feed intake (mg/prawn) over 6 h, of prawns fed diets containing gramine. Gramine content (mg/kg of DM) of the diets is indicated in the label. *n* = 7 for each diet, error bars indicate standard errors.

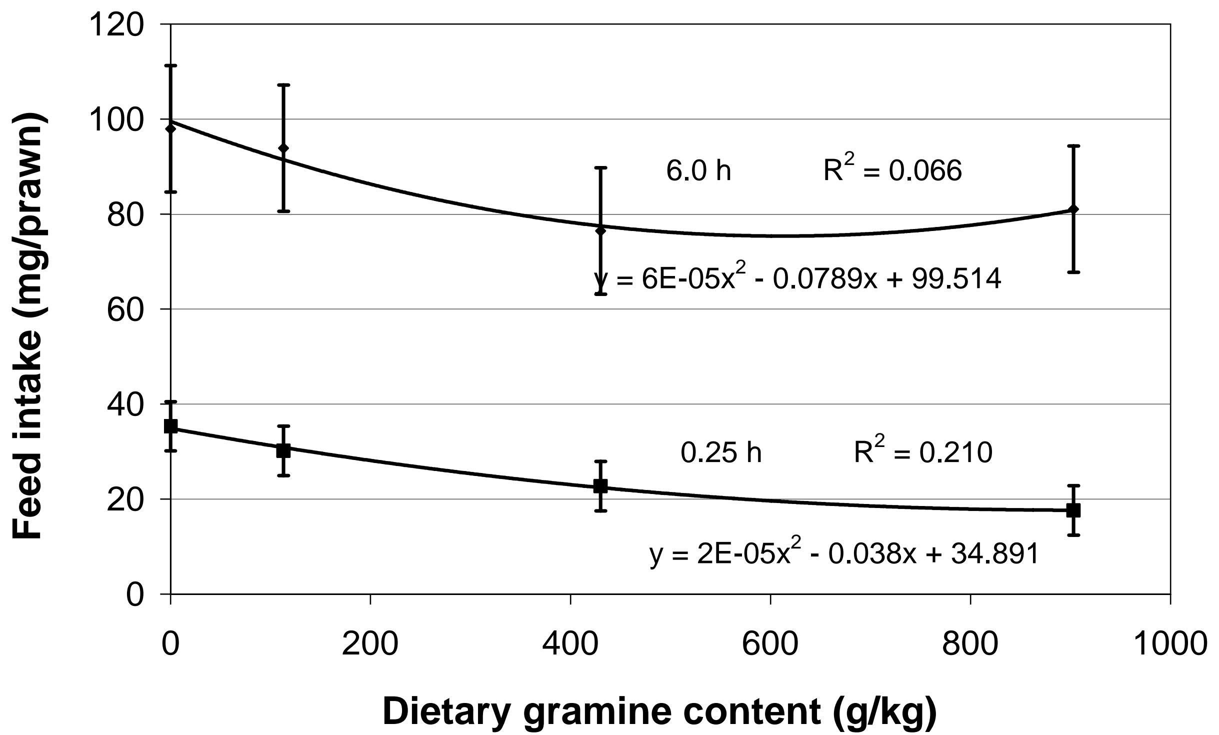

Figure 27.3 Average feed intake of prawns (mg/prawn) fed diets containing varying concentrations of gramine over the first 0.25 h after feed distribution and after 6 h. *n* = 7 at each dietary gramine concentration; error bars show standard error of the mean.

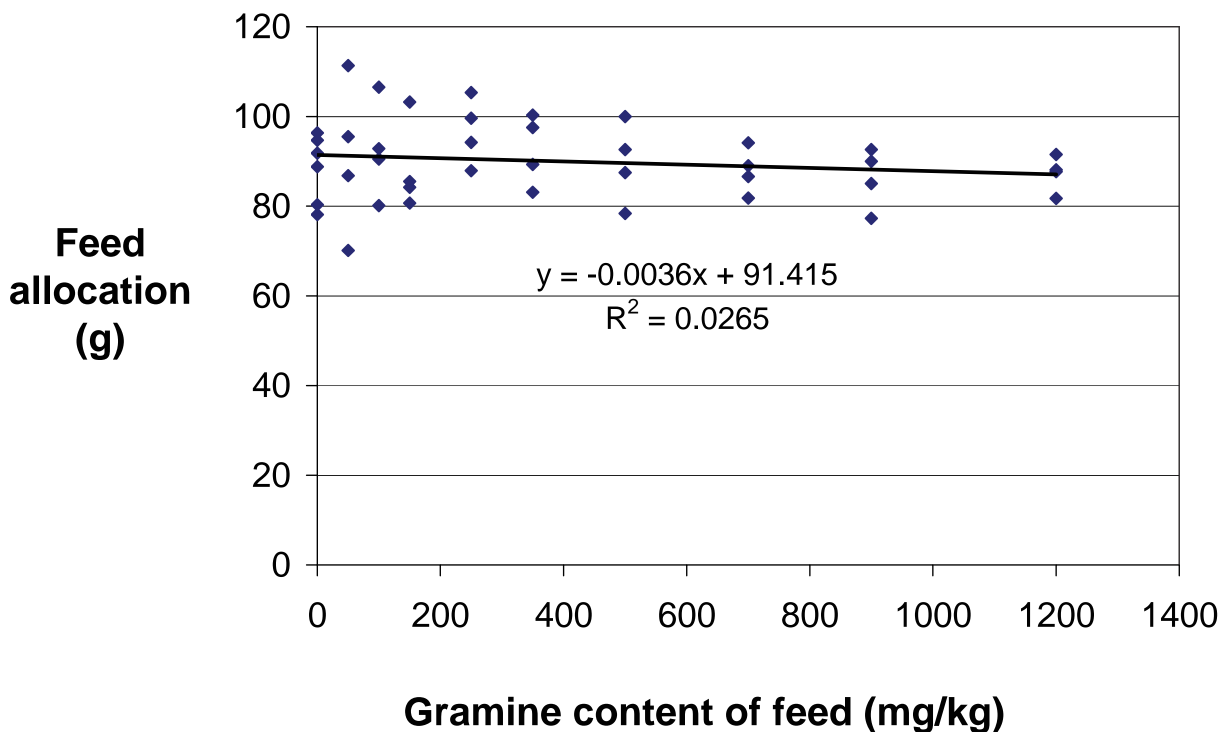

Figure 27.4 Feed allocation over 50 d to tanks of prawns fed a series of feeds containing increasing levels of the lupin alkaloid, gramine.

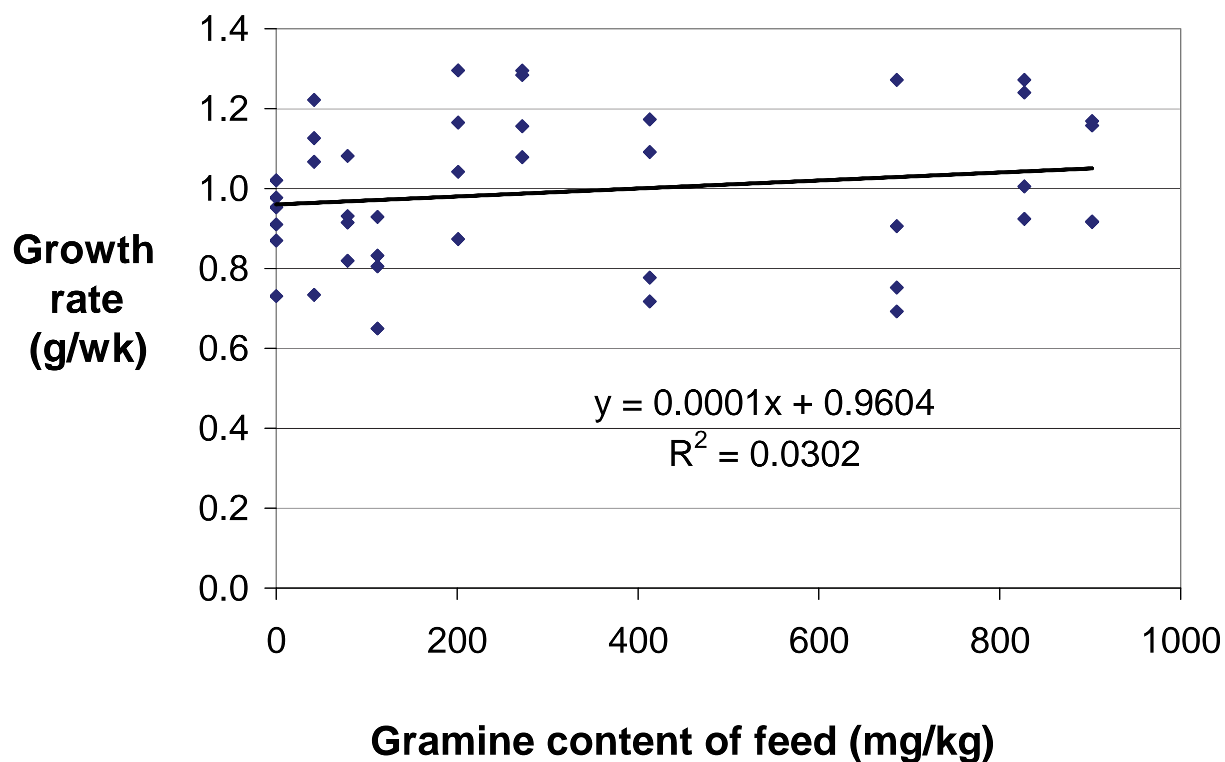

Figure 27.5 Growth response of black tiger prawns over 50 days of feeding on a series of feeds containing increasing levels of the lupin alkaloid, gramine.

28.0 A comparison of the digestibility of lupin kernel meals when fed to rainbow trout (*Oncorhynchus mykiss*), Atlantic salmon (*Salmo salar*) and Black tiger shrimp (*Penaeus monodon*)

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Abstract

This study compared the ingredient digestibilities of a series of lupin (*L. angustifolius*) kernel meals when fed to either Atlantic salmon, Black tiger shrimp or Rainbow trout in three independent studies. Digestibility of the nitrogen (protein) content of the lupin kernel meals was lowest in the Atlantic salmon (0.735 ± 0.036) and highest in the Black tiger shrimp (0.935 ± 0.005). Variability among the nitrogen digestibilities was lowest for the Black tiger shrimp (range 0.928 to 0.950) and highest for the Rainbow trout (range 0.655 to 1.083). Digestibility of the energy content of the lupin kernel meals was lowest in the Rainbow trout (0.605 ± 0.029) and highest in the Black tiger prawns (0.746 ± 0.016). Variability among the energy digestibilities was lowest for the Rainbow trout (range 0.526 to 0.624) and highest for the Atlantic salmon (range 0.599 to 0.753). There was limited correlation between the digestibilities of the ingredients between the three experiments. The strongest correlations were those between the Black tiger shrimp and Atlantic salmon for nitrogen (R^2=0.997). However, a lack of variability in the digestibility values used in this assessment resulted in limited viability of the correlation, with a regression coefficient of –0.03x indicating a lack of response between the two digestibility assessments despite a high-level of linearity in the data. Diet energy digestibilities were generally more poorly correlated than the diet nitrogen digestibilities, with only a single correlation being of any significance (Rainbow trout vs Black tiger shrimp; R^2=0.675). In contrast to earlier comparisons, correlations between Atlantic salmon and rainbow trout digestibilities were consistently poor for both nitrogen and energy digestibilities. The lack consistent correlation between ingredient digestibilities demonstrates the need for such trials to be wholly conducted within the one laboratory to minimise inter-laboratory variance.

28.1 Introduction

Although there is a considerable volume of work on the nutritional value of grain products for both salmonids and shrimp (Kaushik et al., 1995; Refstie et al., 1998; Carter and Hauler, 1999; Sudaryono et al., 1999; Burel et al., 2000; Refstie et al., 2000; Glencross and Hawkins, 2004; Glencross et al., 2004a; 2004b; Smith et al., 2007a; 2007b), there is no published comparison of the nutritional value of the same grain products fed to different animals. The digestible value of lupins for shrimp has been shown to be generally similar to that of most fish species (Smith et al., 2000). Generally the apparent digestibility values of dry matter, protein and energy are all higher in *L. angustifolius* kernel meal relative to that of the whole-seed meal. The digestibility values observed for *L. angustifolius* kernel meal are generally similar to that of soybean meal, with marginally higher apparent protein digestibilities (94% vs 92%), though marginally lower apparent energy digestibilities (68% vs 71%).

Even though rainbow trout, *Oncorhynchus mykiss* and Atlantic salmon, *Salmo salar*, are both from the same family of fish, there have been inconsistent results about the homology in nutritional responses of the two species when fed similar raw materials (Refstie et al., 2000; Glencross et al., 2004a). Studies by Refstie et al. (2000) compared the nutritional responses of Atlantic salmon and rainbow trout when fed soybean meal and noted that the two species had a different growth response to the inclusion of this ingredient. Glencross et al. (2004a) examined the digestibility of lupin and soybean meals, concentrates and isolates when rainbow trout and Atlantic salmon were fed the same diets and when the same faecal collection methods had been used. Although Glencross et al. (2004a) found some differences in the digestibility values for the same ingredients when fed to either species, these authors also found that there was a high degree of correlation in the digestibility values between the two species. In particular there was a high degree of correlation in responses to energy digestibilities, but a less significant correlation in nitrogen digestibilities. Krogdahl et al. (2004) also compared the digestion and utilisation of high and low corn starch diets when fed to both Atlantic salmon and rainbow trout. It was shown that the growth responses of each species were quite similar, as were the energy and protein retention features. Marginal differences in the digestibilities were observed between the rainbow trout and Atlantic salmon. However, differences in diet digestibilities were also noted between fish maintained in either freshwater or seawater fish in this work (Krogdahl et al., 2004).

This study examines a comparison in the digestibility values of series of lupin kernel meals when fed to rainbow trout, *Oncorhynchus mykiss* or Atlantic salmon, *Salmo salar* or Black tiger shrimp, *Penaeus monodon*. The data is derived from three separate studies undertaken by three independent laboratories, each evaluating the same series of lupin kernel meals but with different species or under different water temperatures and/or salinities (Smith et al., 2007a; Chapter 7; Chapter 21). The specific digestibility aspects of the lupin kernel meals being evaluated are not discussed in this chapter as they have been detailed elsewhere. This comparison was done to examine the transferability of data for one species to the other and the robustness of inter-laboratory comparisons of digestibility assessments.

28.2 Materials and Methods

28.2.1 Ingredient and diet development

Separate batches of seed of *Lupinus angustifolius* were collected from the Department of Agriculture and Food's (WA) lupin germplasm and breeding lines, predominantly from the 2003 crop season at Wongan Hills Research Station. Samples of the seed were then split using a small disc-mill and aspirated to separate hulls from kernels. A final manual cleansing of the kernels to remove any remaining hull material was also undertaken on each sample to ensure 100% purity of the kernel preparation. Each kernel sample was then milled using a Retsch rotor mill with a 750 µm screen to create a kernel flour. In addition to the lupin kernel flours, each of the test ingredients used in this study was thoroughly ground so that they passed through a 750 µm hammer mill screen.

The experiment design was based on a diet formulation strategy that allowed for the diet-substitution digestibility method to be used (Aksnes et al., 1996). For the fin-fish, a basal diet was formulated and prepared to include approximately 500 g/kg DM protein, 210 g/kg DM fat and an inert marker (yttrium oxide at 1 g/kg) (Table 28.2). A basal mash was prepared and thoroughly mixed, forming the basis for all experimental diets in this study. The ingredient

of study for each test diet was added at 30% inclusion to a sub-sample of the basal mash (see Table 28.2). Diets were processed by addition of water (about 30% of mash dry weight) to the mash whilst mixing to form a dough, which was subsequently screw pressed using a pasta maker through a 4 mm diameter die. The resultant moist pellets were then oven dried at 70°C for approximately 12 h and then allowed to cool to ambient temperature in the oven. The basal diet was prepared in a similar manner, but without the addition of any test ingredient. An additional reference lupin kernel meal was included in every digestibility study to allow for cross-comparison across all studies. The basal diet and an example test diet formulations and their composition are presented in Table 28.2.

For the Black tiger shrimp the reference diet used in this study (Table 28.3) was formulated to be nutritionally-adequate and attractive to the shrimp, with 390 g/kg crude protein and 100 g/kg total lipid, on DM basis. Micro-nutrients were included at twice the minimum rate in the reference diet to ensure that they were not deficient when diluted with the test ingredients in the test diet formulations. The test diets comprised 50% by weight of the kernel meal ('as used' basis) and 50% by weight of the reference diet mash ('as used') (Table 28.3). The test diets had a similar crude protein content as the Reference diet (range: 380 to 425 g/kg) but slightly less total lipid (~ 90 g/kg). Ytterbium acetate tetrahydrate (99.9%, Aldrich, Sydney, Australia) was included in the feeds as an inert digestibility marker at a rate of 0.5 g/kg. Water was added to the mixed ingredients to form a dough containing 40 to 50% moisture. The dough was extruded twice through a 3 mm die of a meat mincer (Hobart Corporation, Troy, OH, USA) to form spaghetti-like strands which were air dried in a forced-draught cabinet at 40°C, and then re-ground to pass through a 500 µm screen. Additional water was added to the re-ground material and the 'feed' mixed to form a dough again. This dough was extruded twice through the mincer, steamed for 5 min and air dried again before being broken-up into 5 to 10 mm pellets and stored at -5°C until used. This process was found to significantly improve the homogeneity of the feed pellets (Smith and Tabrett, 2004).

28.2.2 Animal handling

Batches of the experimental feeds were sent from Western Australia to the School of Aquaculture – University of Tasmania, at their Launceston laboratory in Tasmania, Australia, who undertook Atlantic salmon digestibility analysis. Batches of the lupin kernel meals were sent from Western Australia to the CSIRO Division of Marine and Atmospheric Research, at their Cleveland laboratory in Queensland, Australia, who undertook Black tiger prawn digestibility analysis.

28.2.2.1 Rainbow trout handling and faecal collection

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*, Pemberton heat-tolerant strain, Western Australia; Molony et al., 2004) were transferred from grow-out ponds to experimental tanks (200 l). Freshwater (salinity < 1 PSU; Dissolved oxygen 7.0 ± 0.5 mg/L) of 16.0 ± 0.1°C (mean ± S.D.) at a flow rate of about 4 l/min was supplied to each of the tanks. Each of the tanks were stocked with 15 trout of 198 ± 33.8 g (mean ± S.D.; n = 40). Treatments were randomly assigned amongst 24 tanks, with each treatment having three replicates. Fish were manually fed the diets once daily to apparent satiety as determined over three separate feeding events between 1500 and 1600 each day. The trout were allowed to acclimatise to the allocated dietary treatment for seven days before faecal collection commenced consistent with earlier studies by this group (Glencross et al., 2005). Faeces were collected using stripping techniques. Stripping techniques were based on those reported by Austreng (1978). After removal of the faeces from the fish, the faecal sample was placed in a small plastic vial and stored in a freezer at -20°C. Stripped

faeces were collected between 0800 and 1000 over a four-day period, with each fish only being stripped twice and not on consecutive days. Faecal samples from different days were pooled within tank, and kept frozen at -20°C before being freeze-dried in preparation for analysis.

28.2.2.2 Atlantic salmon handling and faecal collection

Mixed-sex, diploid, very-late-spring (January) Atlantic salmon smolt were obtained from Mountain Stream Fishery (Targa, Tasmania, Australia) (farm weight estimate, 180 g). Salmon were held at the School of Aquaculture in six 2000-L Rathbun tanks that were each a self-contained partial recirculation system equipped with physical, biological and UV filtration. Water temperature was controlled at 15.0 ± 1.5 °C, salinity at 30 ± 2 ppt and fish were exposed to ambient photoperiod. Water quality was maintained within recommended limits (Tarazona & Munoz, 1995). The fish were acclimated to the systems that were then used to hold the fish for the experiments. During acclimation a commercial salmon feed was hand fed two times per day for 8 weeks.

At the start of the apparent digestibility experiment all diets were hand fed two times per day at 0.6% body weight (BW). The six diets were randomly allocated to one group in each of three time periods. Diets were fed for 9 days and the salmon stripped (Austreng, 1978; Percival *et al.*, 2001) on the morning of day 10. In order to randomise the effects of previous diets the salmon were mixed during reallocation to tanks and fed the commercial diet for a further 18 days. Following initial sampling salmon were reused twice to obtain triplicate samples for each diet.

28.2.2.3 Black tiger shrimp handling and faecal collection

The two digestibility experiments involved the feeding of the reference diet and six lupin kernel meal diets to groups of prawns (mean weight ± SD: Experiment 1 = 23.5 ± 3.8 g , Experiment 2 = 16.6 ± 2.4 g). In both experiments, six tanks, each containing two randomly-selected prawns, were allocated to each dietary treatment. The prawns were placed in the tanks 7 days prior to the start of the faecal collection periods, to adapt to their allocated diet. During the adaptation period the prawns were fed twice daily and no faeces were collected. After the adaptation period, and commencing on a Monday at 06:00 am, the prawns were fed every 6 h, with a 30 second interval between feeding successive tanks. Thirty minutes after the feed was put in the tanks, all the uneaten feed pellets and fragments were removed from the tanks by siphoning and discarded. Thereafter, faeces from individual tanks were collected by siphoning 3 h after feeding and again immediately before feeding. This process ran continuously for 5 d each week until Saturday mornings at 06:00 am. Between Saturday and Monday mornings, the prawns were fed twice daily and no faeces were collected.

The faeces siphoned from the each tank were collected into a 10 L bucket and within 30 min were transferred into a 10 mL centrifuge tube using a wide mouth pipette tip and bulb. The excess water was decanted from the centrifuge tubes after a short settling time. Distilled water was added to the tubes to make the volume up to 10 mL and the tubes centrifuged at 2000 rpm (700 rcf) for 30 sec. The supernatant was decanted off, and the tubes capped and placed in a freezer. Once frozen, the faecal pellet was transferred to a pre-weighed sample vial and stored at -20°C.

This routine was maintained for about 10 weeks in both experiments until at least 2 g dry weight of faecal material (~30 g of wet faeces) had been collected from each tank. This was the amount required for the intended chemical analyses for dry matter (DM), crude protein, energy and ytterbium. At the end of the experiment, faeces were freeze-dried, ground and stored at -20°C.

28.2.3 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet and faecal samples were analysed for dry matter, yttrium (or ytterbium), ash, phosphorus, nitrogen and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total yttrium and phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES) based on the method described by (McQuaker et al., 1979). Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Total lipid content of the diets was determined gravimetrically following extraction of the lipids according to the Folch method. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. Differences in the ratios of the parameters of dry matter, protein, amino acids or gross energy to yttrium, in the feed and faeces in each treatment were calculated to determine the apparent digestibility coefficient (ADC_{diet}) for each of the nutritional parameters examined in each diet based on the following formula (Maynard and Loosli, 1979):

$$ADC_{diet} = 1 - \left(\frac{Y_{diet} \times Parameter_{faeces}}{Y_{faeces} \times Parameter_{diet}} \right)$$

Where Y_{diet} and Y_{faeces} represent the yttrium or ytterbium content of the diet and faeces respectively, and $Parameter_{diet}$ and $Parameter_{faeces}$ represent the nutritional parameter of concern (organic matter, protein or energy) content of the diet and faeces respectively. Digestibility values for each diet are presented in Table 4. The digestibility values for each of the test ingredients in the test diets examined in this study were calculated according to the formulae:

$$Nutr.AD_{ingredient} = \frac{\left(AD_{test} \times Nutr_{test} - \left(AD_{basal} \times Nutr_{basal} \times 0.7\right)\right)}{\left(0.3 \times Nutr_{Ingredient}\right)}$$

Where $Nutr.AD_{ingredient}$ is the digestibility of a given nutrient from the test ingredient included in the test diet at 30%. AD_{test} is the apparent digestibility of the test diet. AD_{basal} is the apparent digestibility of the basal diet, which makes up 70% of the test diet. $Nutr_{Ingredient}$, $Nutr_{test}$ and $Nutr_{basal}$ are the level of the nutrient of interest in the ingredient, test diet and basal diet respectively (Sugiura et al., 1998). All raw material inclusion levels were also corrected for dry matter contribution and the effects that this may have had on the actual ratio of reference diet to test ingredient (Bureau and Hua, 2006).

Digestibilities greater than 100% were not corrected because we consider they are potentially indicative of interactive effects between the diet and test ingredient and should be stipulated as determined.

28.2.4 Statistical analysis

All values are means unless otherwise specified. Correlation analysis was performed using Microsoft Excel. Curve fitting of linear regressed relationships was undertaken using both Microsoft Excel.

28.3 Results

In contrast to the chapter comparing the digestibility of a series of diets and ingredients when fed to Atlantic salmon and Rainbow trout, in the present study while such a comparison could be made between the two fin-fish species, it is not applicable to the shrimp because of the different diets used and the different raw materials applied to the reference diets for each species. Accordingly, only a comparison of the component ingredient digestibilities is examined.

28.3.1 Ingredient digestibilities

There was substantial variation in the digestibility parameters between the three experiments (Table 28.3). Ingredient nitrogen (protein) digestibilities (mean ± SD) of the lupin kernel meals were lowest in the Atlantic salmon (0.735 ± 0.036) and highest in the Black tiger shrimp (0.935 ± 0.005). Variability among the nitrogen digestibilities was lowest for the Black tiger shrimp (range 0.928 to 0.950) and highest for the Rainbow trout (range 0.655 to 1.083). Ingredient energy digestibilities of the lupin kernel meals were lowest in the Rainbow trout (0.605 ± 0.029) and highest in the Black tiger prawns (0.746 ± 0.016). Variability among the energy digestibilities was lowest for the Rainbow trout (range 0.526 to 0.624) and highest for the Atlantic salmon (range 0.599 to 0.753).

There was limited correlation between the digestibilities of the ingredients between the three experiments (Table 28.4). The strongest correlations were those between the Black tiger shrimp and Atlantic salmon for nitrogen (R^2=0.997) (Table 28.4, Figure 28.3). Diet energy digestibilities were generally more poorly correlated than the diet nitrogen digestibilities, with only a single correlation being of any significance (Rainbow trout vs Black tiger shrimp; R^2=0.675) (Table 28.4; Figure 28.1). In contrast to earlier comparisons, correlations between Atlantic salmon and Rainbow trout digestibilities were consistently poor for both nitrogen and energy digestibilities (Table 28.4, Figure 28.2).

28.4 Discussion

This study examined a comparison in the digestible value of a series of lupin kernel meals when fed to Rainbow trout, Black tiger shrimp or Atlantic salmon. The data was derived from three separate studies undertaken by three independent laboratories, each evaluating the same ingredients but with either different species or under different water temperatures (Chapter 7, 11, 21 and Smith et al., 2007a). In the experiments with shrimp, different reference diets were also used and this difference prevents a valid comparison in diet digestibilities across the three species/experiments. However, the diet digestibility comparison builds on from earlier work that examined the digestibility of a series of lupin and soybean products when fed to Rainbow trout and Atlantic salmon by the same group of researchers (Glencross et al., 2004a). Other differences such as water salinity and temperature make the comparisons more difficult but were present because the experiments were not designed as a formal comparison study across species. However, despite these differences, a comparison of the three experiments provides some insight into the scope and limitations of cross-species/experimental comparisons.

28.4.1 Ingredient digestibility effects

As found in earlier comparative studies, there was substantial variation in the digestibility parameters among the three experiments (Glencross et al., 2004a; Chapter 20). Also as in the findings from Chapter 20 and Glencross et al. (2004a), the ingredient nitrogen (protein)

digestibilities were lower in the Atlantic salmon than the Rainbow trout. The high nitrogen digestibilities observed in the Black tiger shrimp is also consistent with other digestibility work with this species and may be an artefact of the settlement collection method used, which has also shown consistently higher nitrogen digestibility values with reduced variability among test ingredients in studies comparing the settlement and stripping techniques used in faecal collection with Rainbow trout (Smith and Tabrett, 2004; Glencross et al., 2005). The variability of the nitrogen digestibility in the Rainbow trout (range 0.655 to 1.083) is consistent with other studies that have shown a similar level of variability (Glencross et al., 2003; 2005; 2007; Chapter 7 and 11). A similar level of variability among the energy digestibilities was also observed for the Rainbow trout (range 0.526 to 0.624).

The limited correlation between either the nitrogen or energy digestibilities of the ingredients, between the three experiments, is consistent with earlier studies (Chapter 20). However this contrasts those results reported by Glencross et al. (2004a) who showed strong correlation between ingredients fed to Atlantic salmon and Rainbow trout, particularly so for energy digestibilities which had a high-degree of variation in the digestibilities of each of the test ingredients. However, a key difference between the present study and that of Glencross et al. (2004a) is the limited variability in composition and digestibilities of the ingredients used in the present study. This lack of variability significantly weakens the potential capacity of cross-correlations. The level of variability among the nitrogen digestibilities for the black tiger shrimp (range 0.928 to 0.950) is a classic example if this and the effects are clearly demonstrated in Figure 28.3.

The strongest correlations were those between the Black tiger shrimp and Atlantic salmon for nitrogen (R^2=0.997). However, a regression coefficient of -0.03x indicates that despite strong linearity in this relationship that there is little response (either negative or positive) between the digestibility coefficients between the two species (Figure 28.3). Although the diet energy digestibilities were generally more poorly correlated than the diet nitrogen digestibilities, the single correlation of significance (Rainbow trout vs Black tiger shrimp; R^2=0.675) (Table 28.4; Figure 28.1) was also probably the most meaningful correlation in this whole study. In this regard, not only was a high correlation (R^2=0.675) observed, but a regression coefficient of 2.114x also indicates a strong positive response between the digestibility coefficients between the two species (Figure 28.3).

In contrast to earlier comparisons, correlations between Atlantic salmon and Rainbow trout digestibilities were consistently poor for both nitrogen and energy digestibilities (Glencross et al., 2004a; Chapter 20). These correlations may have been weakened by the inter-laboratory differences in collection and analytical methods, which in contrast to some earlier studies (Glencross et al., 2004a) were not standardised but were similar. An additional difference to the study of Glencross et al. (2004a) was that the present study used stripping techniques compared to the settlement faecal collection methods used in other study.

28.4.2 Conclusions

The findings of this study show that there are considerable differences between different laboratories assessing the same raw materials, albeit in different animal species, in different water salinities and at different temperatures. This finding supports earlier assertions that the most robust comparisons are likely to be ones made within the same laboratory as demonstrated by the comparison of the findings from the present study compared with those of Glencross et al. (2004a). Although the differences among these inter-laboratory studies make it difficult to

confirm digestibility differences or similarities of different grain products by the three species this does not diminish the need to a robust intra-laboratory comparison to assess commonality in nutritional value of raw materials for multiple aquaculture species.

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Table 28.1 Nutrient composition of the experimental ingredients used with trout and Atlantic salmon (all values are g/kg DM unless otherwise indicated).

Actual	Fishmeal	Gungurru	Kalya	Tanjil	Mandelup	Coromup	Myallie	Wonga	Wodjil
Dry Matter (g/kg)	931	914	916	920	916	919	921	916	909
Protein (N x 6.25)	749	538	508	497	505	505	452	487	537
Fat	87	63	60	68	62	66	73	70	77
Ash	161	38	39	35	35	36	33	34	44
Carbohydrate [a]	3	361	392	400	397	393	441	409	342
Energy (MJ/kg DM)	20.52	20.62	20.54	20.58	20.53	20.44	20.26	20.62	21.10
Arginine	41	66	59	54	52	59	48	54	53
Cysteine	10	7	8	7	8	6	6	7	–
Histidine	13	11	11	10	9	10	8	9	14
Isoleucine	29	20	19	18	18	17	16	18	21
Leucine	56	38	36	32	33	34	30	33	44
Lysine	55	24	23	20	22	23	20	21	27
Methionine	21	3	2	3	3	3	2	3	5
Phenylalanine	30	21	19	18	18	18	16	18	22
Threonine	32	18	17	17	18	19	16	16	19
Valine	33	18	17	17	18	18	15	17	17

Fish meal: Chilean anchovy meal and Australian feed grade wheat, Skretting Australia, Cambridge, TAS, Australia. All *L. angustifolius* Kernel Meals sourced from the germplasm collection of, Department of Agriculture and Food, South Perth, WA, Australia.

Table 28.2 Formulations of the experiment diets used with rainbow trout and Atlantic salmon (all values are g/kg).

	Reference	Mandelup	Gungurru	Tanjil	Kalya	Coromup	Myallie	Wonga	Wodjil
Ingredient									
Fishmeal	700	490	490	490	490	490	490	490	490
Fish oil	150	105	105	105	105	105	105	105	105
L. angustifolius cv. Mandelup kernel meal		300							
L. angustifolius cv. Gungurru kernel meal			300						
L. angustifolius cv. Tanjil kernel meal				300					
L. angustifolius cv. Kalya kernel meal					300				
L. angustifolius cv. Coromup kernel meal						300			
L. angustifolius cv. Myallie kernel meal							300		
L. angustifolius cv. Wonga kernel meal								300	
L. luteus cv. Wodjil kernel meal									300
Wheat flour	144	100.8	100.8	100.8	100.8	100.8	100.8	100.8	100.8
Vitamin and mineral premix	5	3.5	3.5	3.5	3.5	3.5	3.5	3.5	3.5
Yttrium oxide	1	0.7	0.7	0.7	0.7	0.7	0.7	0.7	0.7

[a] From *L. luteus* (yellow lupins).

[b] From *L. angustifolius* (Sweet lupins).

* Vitamin and mineral premix includes (IU/kg or g/kg of premix): Vitamin A, 2.5MIU; Vitamin D3, 0.25 MIU; Vitamin E, 16.7 g; Vitamin K,3, 1.7 g; Vitamin B1, 2.5 g; Vitamin B2, 4.2 g; Vitamin B3, 25 g; Vitamin B5, 8.3; Vitamin B6, 2.0 g; Vitamin B9, 0.8; Vitamin B12, 0.005 g; Biotin, 0.17 g; Vitamin C, 75 g; Choline, 166.7 g; Inositol, 58.3 g; Ethoxyquin, 20.8 g; Copper, 2.5 g; Ferrous iron, 10.0 g; Magnesium, 16.6 g; Manganese, 15.0 g; Zinc, 25.0 g.

Table 28.3 Formulations and composition of the experiment diets used with black tiger shrimp (all values are g/kg).

Ingredient	Reference	Myallie	Kalya	Mandelup	Tanjil
L. angustifolius kernel meal cv Myallie	–	500	–	–	–
L. angustifolius kernel meal cv Kalya	–	–	500	–	–
L. angustifolius kernel meal cv Mandelup	–	–	–	500	–
L. angustifolius kernel meal cv Tanjil	–	–	–	–	500
Flour (wheat)[1]	376	188	188	188	188
Gluten (Wheat, 76% CP) [2]	120	60	60	60	60
Fishmeal (Peruvian 68% CP) [3]	200	100	100	100	100
Squid meal [3]	100	50	50	50	50
Crustacean meal [4]	100	50	50	50	50
Cod liver oil[5]	40	20	20	20	20
Soybean lecithin (70% lipid)[6]	30	15	15	15	15
Cholesterol (100%)[7]	10	5	5	5	5
Vitamin mix[8]	20	10	10	10	10
Sodium ascorbyl-2-polyphosphate (Stay C)[9]	2	1	1	1	1
Astaxanthin (Carophyll Pink 10%)[10]	1	0.5	0.5	0.5	0.5
Ethoxyquin (Banox E) [9]	0.4	0.2	0.2	0.2	0.2
Ytterbium acetate tetrahydrate[11]	0.5	0.5	0.5	0.5	0.5

[1] Flour, White Wings, Brisbane, Queensland, Australia
[2] Wheat gluten (76% CP), Janbak Industries Pty Ltd, Brisbane, Queensland.
[3] Fishmeal and squid meal supplied by Ridley Aquafeeds Pty Ltd, Narangba, Queensland
[2] Corn starch, Janbak Industries Pty Ltd, Brisbane, Queensland, Australia.
[4] Crustacean meal, Inual, Santiago, Chile, supplied by Ridley Aquafeeds
[5] Melrose Laboratories, Box Hill, Victoria, Australia.
[6] Supplied by Janbak Industries Pty Ltd, Brisbane, Queensland.
[7] Ajax Chemicals, Sydney, NSW, Australia
[8] Vitamin mix. (Conklin,1997), supplied by Rabar Pty Ltd, Beaudesert, Queensland
[9] Adisseo Australia, Carole Park, Qld
[10] Donated by DSM Nutritional Products Australia Pty Ltd, Sydney, NSW.
[11] Aldrich, Sydney NSW.

Table 28.4 Digestibility (%) specifications of lupin kernel meals for each of the test species.

	Mandelup	Gungurru	Tanjil	Kalya	Myallie	Coromup	Wonga	Wodjil	Mean	SEM
Ingredient Digestibility – Rainbow trout										
Energy	0.597	0.601	0.526	0.573	0.542	0.624	0.578	0.774	0.605	0.029
Protein	0.655	0.919	0.858	1.002	0.996	1.083	0.928	0.887	0.916	0.045
Ingredient Digestibility – Atlantic salmon										
Energy	0.683	0.599	0.753	–	0.655	0.715	–	–	0.681	0.026
Protein	0.603	0.784	0.776	–	0.799	0.714	–	–	0.735	0.036
Ingredient Digestibility – Black tiger prawns										
Energy	0.734	–	0.728	0.758	0.696	–	0.759	0.799	0.746	0.016
Protein	0.933	–	0.928	0.950	0.927	–	0.946	0.938	0.935	0.005

Table 28.5 Summary of cross-species correlations between each of the studies for ingredient digestibilities of nitrogen (protein) and energy.

	RT	AS	BTP	RT	AS	BTP
	Nitrogen Digestibilities			*Energy Digestibilities*		
RT	–	–	–	–	–	–
AS	0.419	–	–	0.101	–	–
BTP	0.106	0.997	–	0.675	0.366	–

RT: Rainbow trout, AS: Atlantic salmon, BTP: Black tiger prawns.

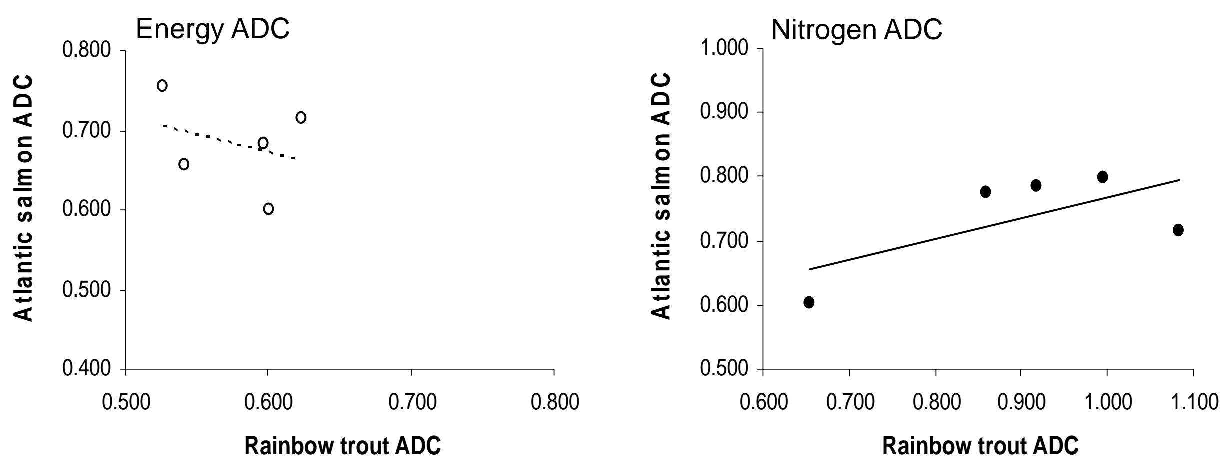

Figure 28.1 Correlations among nitrogen digestibilities of the same lupin kernel meals when fed to either Atlantic salmon or rainbow trout. Equations for regression function are: Energy digestibility, $y = -0.4481x + 0.9402$, $R^2 = 0.1013$. Nitrogen digestibility, $y = 0.3221x + 0.4446$, $R^2 = 0.4186$.

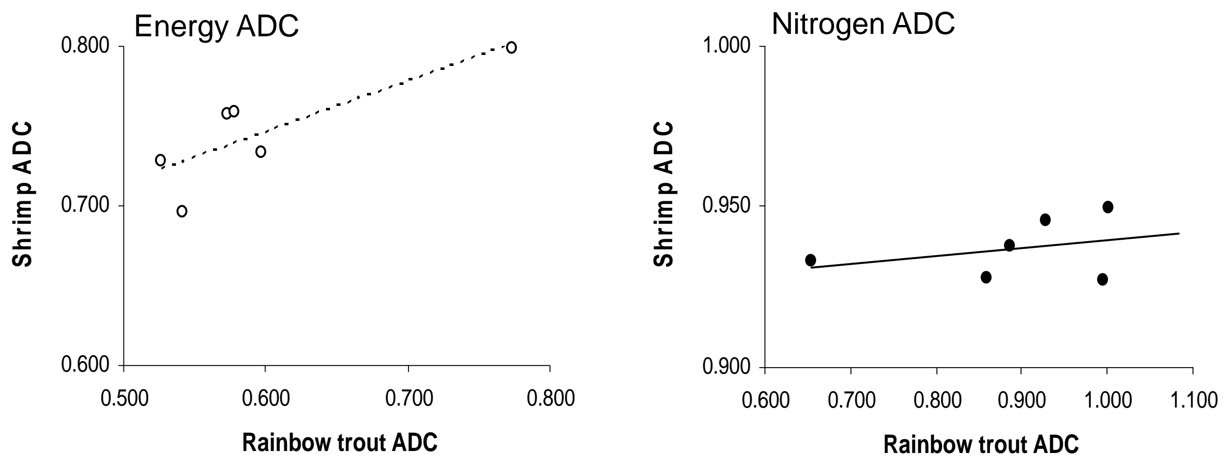

Figure 28.2 Correlations among protein digestibilities of the same lupin kernel meals when fed to either Black tiger shrimp or rainbow trout. Equations for regression function are: Energy digestibility, y = 0.3191x + 0.5548, R^2 = 0.6746. Nitrogen digestibility, y = 0.0242x + 0.9155, R^2 = 0.106.

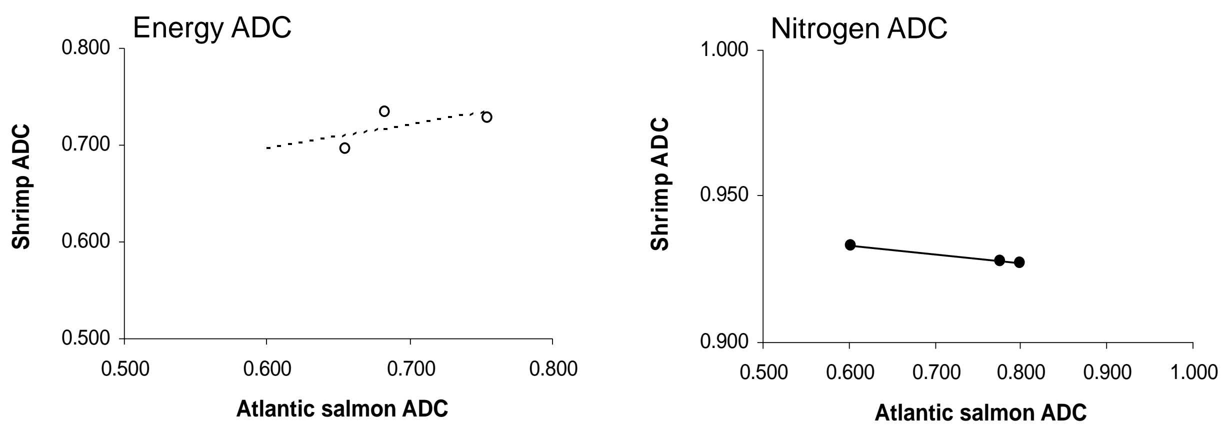

Figure 28.3 Correlations among nitrogen digestibilities of the same lupin kernel meals when fed to either Atlantic salmon or rainbow trout. Equations for regression function are: Energy digestibility, y = 0.2406x + 0.5516, R^2 = 0.3663. Nitrogen digestibility, y = -0.03x + 0.9511, R^2 = 0.9973.

29.0 Effect of lupin kernel meal inclusion on extruded salmonid pellet characteristics

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Abstract

This study examined the influence of different lupin varieties and their inclusion levels on the physical features of an extruded fish diet. Lupin (*Lupinus angustifolius* and *Lupinus luteus*) kernel meals of several different cultivars were included into mashes of a fish diet formulation at 10%, 20% and 30% on a weight-for-weight basis. Soybean meal was also included in a series of diets on similar basis as a reference. An unadulterated basal mash was also used as a 0% inclusion reference. The diet mashes were extruded through a laboratory-scale APV 19:45 twin-screw extruder. The operating parameters and screw-configuration were kept constant for each diet treatment. The addition of water was also kept constant for each treatment. Each diet was run through the extruder for 5 minutes before a sample of the pellets was collected for quality analysis. Pellets were subjected to a range of quality analyses; including radial expansion, bulk density, vacuum oil uptake, sink rate and shear strength. The inclusion of lupin kernel meal (either *L. angustifolius* or *L. luteus*) was found to significantly increase bulk density, sink rate and shear strength of the pellets. With this increase in lupin kernel meal inclusion level a concomitant decline in vacuum oil uptake and radial expansion was also observed. Similar responses were also observed with an increase in the inclusion of soybean meal. Most relationships were not linear with inclusion level, but curvilinear, indicating that there were optimal inclusion levels based on the other raw materials present in each formulation. Assessment of the diet mashes using a Rapid Visco Analyser (RVA) showed that the inclusion of lupin kernel meals increased the rate and degree of viscosity compared to a similar inclusion level of soybean meal. The inclusion of lupin kernel meals in the diet mash was also observed to improve the water holding capacity of the extrudate, which has important implications for the reduction in extruder wear. Key features of the inclusion of lupin kernel meals on the pellet quality parameters were an improved pellet hardness and moisture retention.

29.1 Introduction

Modern aquaculture feeds are manufactured almost exclusively using extrusion technology (Hilton et al., 1981; Jeong et al., 1991; Allan and Booth, 2004). Because the chemical and physical processes that occur during extrusion are considerably different from those encountered during screw-press or steam-pelleting arrangements, it is important to examine the effects of certain raw materials on the extrusion process (Booth et al., 2002; Cheng and Hardy, 2003; Aslaksen et al., 2006). One of the key features of the extrusion process is the gelatinisation and expansion of the starch content of the feed, which has both physical and nutritional benefits (Bergot et al., 1983; Jeong et al., 1991; Romarheim et al., 2005; Kim et al., 2006). Irrespective of the potential effect of high-inclusion levels nutritionally, if certain inclusion levels of raw

materials adversely affect the physical or processing properties of a feed then these processes, rather than the nutritional ones become the limiting constraints to using certain raw materials (Allan and Booth, 2004; Kim et al., 2006; Knudsen et al., 2006; Overland et al., 2007).

The physical properties required of modern aquaculture feeds are also somewhat different to those demands placed on other feed types (Evans, 1999; Overland et al., 2006). Features such as oil absorption capacity; density, durability/hardness and sinking rates are some of such features. The water absorption capacity of the feed mash is also an important feature as it can have significant implications for reducing the depreciation rate of the extrusion equipment, with higher water holding capacity in the mash decreasing the wear of the equipment and reducing the depreciation rate accordingly (Rokey, 2005).

In an effort to reduce feed ingredient risk associated with the production of salmonid feeds, there has been pressure to reduce reliance on fishmeal as a primary protein source (Naylor et al., 2000). Lupin (*Lupinus* spp.) meals are one ingredient that have been shown to provide some potential as a useful feed ingredient in fish diets and are being used in commercial diets in increasing quantities (Burel et al., 1998; Allan and Booth, 2004; Glencross et al., 2004; 2005).

This study evaluates of the influence of several cultivars/varieties of *Lupinus angustifolius* and *Lupinus luteus* kernel meals when included into an extruded reference salmonid formulation. Key physical attributes, such as bulk density, oil absorption, pellet hardness, pellet expansion and sink rates are all examined with respect to several inclusion levels of each kernel meal variety and other key feed grain protein sources. The influence of each raw material on water absorption in the feed mash is also examined.

29.2 Methods

29.2.1 Ingredient and diet preparation

Single crop batches of seed of several *Lupinus angustifolius* cultivars were used in this study. Samples of the seed were dehulled using a SKV abrasive dehuller, followed by differential density aspiration to separate hulls and kernels, before a final manual removal of any remaining hull material. Each of the test ingredients was thoroughly ground using a Retsch™ hammermill such that they passed through a 750 µm screen. The composition and source of all of the ingredients used are presented in Table 29.1.

The experiment design was based on a basal diet formulation to which graded amounts (10%, 20% or 30%) of each test material were added. For this, a single two tonne batch of basal mash was formulated and prepared based on nutritional specifications of approximately 500 g/kg DM protein, 210 g/kg DM fat and 100 g/kg of starch. The same formulation and batch of materials was used for all diets (Table 29.2).

Each of the experimental diets was thoroughly mixed as 10 kg batches using an upright Hobart mixer. No oil or water was added during the mixing phase. Following mixing, the diets were extruded using an APV MFP19:25 laboratory-scale extruder.

29.2.2 Diet extrusion

A laboratory-scale, twin-screw extruder (APV MFP19:25; APV-Baker, Peterborough, United Kingdom), with intermeshing, co-rotating screws was used to process all diets in this study. The barrel was a smooth-walled, open-clam design with twin-screws each with dimensions of 36 x

450 mm (diameter x length). The screw configuration was composed of a series of intermeshing feed screws (FS), forwarding paddles (FP) and lead screws (LS) arranged according to defined barrel diameters (D) such that overall configuration was from the drive end: 16D FS, 2D FP, 1D FS, 2D FP, 1D LS, 1D FP, 2D LS: to the die. A single 2.4 mm diameter cylindrical die tapered at a 67° angle with a land length of 3 mm was used. A dry feed rate of the mash into the barrel was delivered at around 8 to 9 kg/h. Barrel temperatures were set for each of the four zones from drive to die at 70°C, 80°C, 100°C and 110°C respectively. Each diet was extruded using the same temperature parameters (Sorensen et al., 2002). Water was peristaltically pumped (Watson-Marlow 504U, Falmouth, England) into the barrel at approximately 1800 mL/min. Water addition was also kept constant among diets (Lam and Flores, 2003). Product temperature was measured at each of the four zones and die during a product run. Pressure at the die block and drive torque was also monitored every five minutes. Feeds were extruded through the machine at ~250 rpm to obtain a target die pressure of around 250 psi. Pre-conditioning and steam injection were not used during the process. Pellets were cut into 4 to 5 mm lengths using a four-bladed variable speed cutter onto a large aluminium oven trays (650 x 450 x 25 mm, length x width x depth), which were subsequently used for drying of the pellets at 65°C for 12 h. Approximately 2 kg batches of each diet were dried for further processing and evaluation. Operational parameters and extrusion configurations were maintained constant for all test diets.

29.2.3 Pellet evaluation

Following drying all pellets were stored at 4°C. Unless otherwise stated, all measurements were undertaken at room temperature. All measurements were performed in duplicate unless otherwise stated (Gleeson et al., 1999).

29.2.3.1 Vacuum infused oil uptake

Samples of the pellets (100 g) from each treatment were warmed in a drying oven at 60°C for 1 hr prior to being placed in a mixer (Kambrook, Huntingdale, Australia) and an excess (~50 g) of heated (60°C) fish oil added whilst mixing. After mixing for 1 minute the pellets were transferred to a beaker and the beaker placed within the vacuum chamber of a freeze drier. The vacuum chamber was slowly evacuated of air until all visible signs of air escaping from the pellets were observed to cease. Once all visible signs of air escaping had ceased, the vacuum chamber was re-equilibrated to atmospheric pressure and the oil was observed to infuse into the pellet. The pellets were then removed from the beaker and excess oil removed by placing the pellets on absorbent paper towelling. After all excess oil had been removed the final weight of the oil infused pellets was then determined and the relative oil uptake calculated.

29.2.3.2 Radial expansion

The diameters of ten pellets from each treatment were measured using digital vernier callipers (Kingchrome, Robina, Australia) to the nearest 0.01 mm. The mean diameter of the pellets from each treatment was then expressed relative to the die aperture (2.4 mm) as a percent expansion (Gleeson et al., 1999).

29.2.3.3 Bulk density

Bulk samples of the dry pellets post-vacuum coating with their prescribed oil allotment (Table 29.2), were placed within a 100 mL measuring flask and their weight determined. The bulk density was then calculated based on the weight of this volume of the pellets and expressed as g/L (Gleeson et al., 1999).

29.2.3.4 Pellet hardness/ Shear strength

The hardness of the pellets from each treatment was assessed based on the force to shear a pellet across their lateral diameter. The assessment was made using a Stable Microsystems TA-XT2 texture meter (Arrow Scientific, Leichhardt, Australia) with a 15,000 g load-cell and a utility knife blade as the cutting edge. Nine pellets from each treatment were assessed for their hardness, with three random allocations of three pellets through time to avert any effect of cutting blade sharpness that may have occurred over time. The force to shear the pellets was measured as grams of pressure as compression. The texture analyser was set with a pre-test speed of 2 mm/s with a test speed of 0.1 mm/s (Gleeson et al., 1999). The blade was set to pass a maximum distance of 2 mm and trigger at a contact pressure of 10 g. Shear strength was defined as the peak force at breaking of the pellet.

29.2.3.5 Sink rate

Ten pellets from each treatment were individually placed at the surface of a 1000 mL measuring flask containing 1000 mL of freshwater and the time taken to reach the bottom measured using a digital stop-watch. The time taken for each pellet to sink to the bottom of the flask, as a function of the distance, was then calculated to provide a rate of cm/s. Pellets that did not sink were given a zero score, with all other measurements being determined as negative numbers (Gleeson et al., 1999).

29.2.4 Mash moisture holding capacity

An approximate 5 g sample of the premixed mash was accurately pre-weighed into a centrifuge (Hettich Universal, Tuttlingen,Germany) tube and then 10 mL of water added and the tube vortexed for 30 seconds. The tube was then allowed to sit for 60 seconds before being centrifuged at 1000 x *g* for 60 seconds. The resultant supernatant was then decanted from the tube and the tube and its contents re-weighed. The resultant weight gain of the tube contents and the water retained as a function of the dry matter content of the mash was then calculated. Each treatment was assessed in triplicate.

29.2.5 Rapid viscosity analysis

Samples of the diet mashes were evaluated for their pasting characteristics using a Rapid-Visco-Analyser (RVA; Newport Scientific, Warriewood, NSW, Australia) (Whalen et al., 1997). Samples were added to a dry sample vessel at 3.5 g of dry matter with 22 g of total water content. A standard 1 program (2 min at 50°C, ramping to 95°C over 3 min, hold at 95°C for 5min, before reducing to 50°C over 3 min) was run to examine the pasting characteristics of each sample. Key features to be examined where the time of first increase in viscosity, peak viscosity, breakdown viscosity and end viscosity (Masson and Hoseney, 1986; Wrigley et al., 1996; Whalen et al., 1997).

29.2.6 Chemical and digestibility analysis

All chemical analyses were carried out by NATA (National Association of Testing Authorities) accredited analytical service providers (Chemistry Centre (WA), East Perth, WA, Australia). Diet samples were analysed for dry matter, yttrium, ash, phosphorus, total lipids, nitrogen, amino acids and gross energy content. Dry matter was calculated by gravimetric analysis following oven drying at 105°C for 24 h. Total phosphorus concentrations were determined after mixed acid digestion using inductively coupled plasma atomic emission spectrophotometry (ICP-AES)

based on the method described by. Protein levels were calculated from the determination of total nitrogen by Leco auto-analyser, based on N x 6.25. Amino acid composition of samples was determined by an acid hydrolysis prior to separation via HPLC. The acid hydrolysis destroyed tryptophan making it unable to be determined. Crude fat content of the diets was determined gravimetrically following extraction of the lipids using chloroform:methanol (2:1) method. Gross ash content was determined gravimetrically following loss of mass after combustion of a sample in a muffle furnace at 550°C for 12 h. Gross energy was determined by adiabatic bomb calorimetry. All analyses were done in accordance with guidelines specified by AOAC (2005).

29.2.7 Statistical analysis

All figures are mean ± SE unless otherwise specified. Effects of grain type and inclusion level were examined by MANOVA using the software package Statistica (Statsoft®, Tulsa, OA, USA). Levels of significance were determined using Tukeys HSD test, with critical limits being set at $P < 0.05$. Effects of inclusion level of meal on key performance parameters were examined by linear and non-linear regression modelling using Excel.

29.3 Results

29.3.1 Ingredient composition

Each of the lupin kernel meals used in this study varied subtly in their composition (Table 29.1). Crude protein levels ranged from 421 g/kg in the Gungurru to 567 g/kg in the Wodjil. As protein varied in each of the lupin kernel meals, lipid and ash contents remained relatively constant but the carbohydrate content varied to reciprocate the changes in protein concentrations. Gross energy was relatively constant ranging from 20.7 to 21.5 MJ/kg DM. There was little variability noted in the amino acid composition among each of the lupin kernel meals.

29.3.2 Feed extrusion

With increasing inclusion levels of each of the lupin kernel meals a significant increase in bulk density, sink rate and shear strength was observed (Table 29.3). The highest bulk density was observed with a 30% inclusion of Mandelup. Bulk densities of most lupin kernel meal treatments increased with higher inclusion levels (Figure 29.1). This differed from that observed with soybean, which had its greatest effect on bulk density at 20% inclusion and at higher inclusion levels the bulk density reduced. This effect was only observed with the Wodjil variety of lupin kernel meals and none of the *L. angustifolius* lupin varieties. The fastest sink rate was observed with a 20% inclusion level of W2173, though at higher inclusion levels the pellets floated. In most cases, lupin kernel meals produced pellets that had faster sink rates than that observed with similar inclusion levels of soybean meal (Figure 29.4). The greatest shear strength was observed with 30% inclusion level of Myallie-C. The lowest shear-strength of all treatments was observed for the basal diet. Comparatively, all lupin kernel meals produced pellets with greater shear strength than that observed with a similar inclusion level of soybean meal (Figure 29.5).

With increasing inclusion levels of each of the lupin kernel meals a significant decrease in vacuum oil uptake and radial expansion was observed (Table 29.3). However, for both parameters the effect of lupin inclusion level was not linear, with some 30% lupin inclusion treatments producing greater vacuum oil uptakes and greater radial expansion than the same lupin varieties included in diets at 20% (Figures 29.2 and 29.3).

29.3.3 Mash water holding capacity

With increasing inclusion level of lupin kernel meal and soybean meal the feed mash had an increasing its water holding capacity. The effect was strongest for all *L. angustifolius* lupin kernel meal varieties and weakest for the *L. luteus* (cv. Wodjil) variety. A significant difference between the *L. angustifolius* and the reference mash, and the other grain products, was observed at the 10% inclusion level. At the 20% inclusion level there was little variability among the different lupin kernel meals and the soybean meal. At 30% inclusion both the *L. angustifolius* kernel meal and the soybean meal held significantly more water in the mash than the *L. luteus* kernel meal. There was little variation on water holding capacity effects among the different varieties of *L. angustifolius* kernel meals (Figure 29.6).

29.3.4 Rapid viscosity analysis

The inclusion of *L. angustifolius* (cv. Myallie-C) kernel meal in the mash increased the rate of gelatinisation (as measured by the first increase in viscosity) and the peak viscosity during the RVA analysis. With an increase in lupin kernel meal inclusion there was an increase in the peak viscosity, but not the rate of gelatinisation. The end viscosity at both lupin kernel meal inclusion levels was similar to that of the reference mash (Figure 29.7). There was significant variability in the rate of gelatinisation and peak viscosity among the different varieties of *L. angustifolius* kernel meals when included in the mash at 20% (Figure 29.8). All lupin varieties accelerated the gelatinisation process and also increased the peak viscosity. In some cases there was also an increase in the end viscosity as a function of the inclusion of some varieties of lupin kernel meal, such as Gungurru or W2173.

Inclusion of *L. luteus* cv Wodjil increased the rate of gelatinisation and the peak viscosity during the RVA analysis (Figure 29.9). With an increase in *L. luteus* kernel meal inclusion there was no increase in the peak viscosity, or the rate of gelatinisation, with a maximal effect already noted at the 10% inclusion level. The end viscosity at both inclusion levels was lower to that of the reference mash, with the greater the inclusion level resulting in lower end viscosities (Figure 29.9).

Inclusion of soybean meal reduced both the rate of gelatinisation and the peak viscosity during the RVA analysis (Figure 29.10). With an increase soybean meal inclusion there was a reduction in the peak viscosity, the rate of gelatinisation and the end viscosity of the mash. The end viscosity at both inclusion levels was lower to that of the reference mash, with the greater the inclusion level resulting in lower end viscosities (Figure 29.10).

29.4 Discussion

Irrespective of the nutritional value of a raw material, if it cannot be functionally included in a feed with the physical properties required to optimise its delivery to a given species, then its value as a raw material is significantly diminished (Hilton et al., 1981). For modern extruded feeds a range of physical properties of the feed pellets are required to optimise the feed delivery process (Evans, 1999). These features included an ability to bind within a pellet matrix, to allow for some expansion to assist both the gelatinisation of starch and also allow the expansion of the product with some inherent porosity. This porosity aiding both the management of sinking rates of the pellets and also the ability to vacuum infuse liquids, such as fish oil into the pellet (Sopade et al., 2006; Overland et al., 2007). The product should also resist crushing and be resilient to fracturing, features most easily assessed by their shear strength (Evans, 1999). By

assessing the effects that certain raw materials have on such physical properties of extruded products, the potential to manage such features through raw material choice is improved (Hilton et al., 1983; Allan and Booth, 2004; Barrows et al., 2007).

29.4.1 Effects of lupin kernel meals on feed extrusion

The addition of lupin kernel meals to an extruded reference diet produced a range of effects, which varied both depending on the inclusion level of the lupin kernel meal and also the variety of the kernel meal included. The varietal inclusion effect was observed both at the species and cultivar level. Most notably, with an increasing inclusion level of each of the lupin kernel meals, a significant increase in bulk density, sink rate and shear strength was observed (Table 29.3). The highest bulk density was observed with a 30% inclusion of Mandelup. This high bulk density with the inclusion of the Mandelup variety is concomitant with the high level of NSP present in this variety (Smith et al. 2007). While bulk densities of most lupin kernel meal treatments increased with higher inclusion levels, on almost a linear basis, this differed from that observed with soybean, which had a limited increase in bulk density at inclusion levels higher than 20% (Figure 27.1). Despite near linear effects of inclusion on the bulk density of pellets, the response of pellet expansion to increases in the inclusion levels of lupins was clearly non-linear (Figure 29.2). *L. luteus* produced the least expanded pellets at a 20% inclusion level, but the 30% inclusion level had an expansion level similar to that observed of the 10% inclusion level. Consistent with the limited variability observed among the different *L. angustifolius* cultivars on pellet bulk density, there was also limited variability in pellet expansion among the different *L. angustifolius* cultivars. These observed effects are similar to the responses reported by Gleeson (1999), when comparing single inclusion levels (~30%) of a lupin product with soybean meal in diets for Atlantic salmon.

With increasing inclusion levels of each of the lupin kernel meals a significant decrease in vacuum oil uptake and radial expansion was observed (Table 29.3). This has important implications for the development of high-fat fish feeds, which require the vacuum infusion of high levels of lipid, post extrusion and this lack of expansion and poor vacuum oil uptake may limit high inclusion levels of lupins. However, the potential to counter this effect by altering the level of starch inclusion in the diet was not examined and may be an option to allow for the required amount of expansion and still accommodate a high inclusion level of lupin kernel meals. More work on this aspect of raw material functionality is required.

Pellet sink rates were also variably affected by both type of grain meal and also the cultivar of *L. angustifolius* used. Despite similar effects of each of the different *L. angustifolius* cultivars on bulk density substantially different pellet sink rates were observed supporting that pellet sink rate and bulk density may not necessarily be directly related all of the time. The responses of pellet sink rates were in some instances non-linear, but following a critical inclusion level a close to linear effect on pellet sink rates were generally observed.

Pellet hardness was dramatically affected by the inclusion of each of the grain meals. Inclusion of *L. angustifolius* kernel meal had the most pronounced effect on pellet hardness, though there was substantial variability among the different *L. angustifolius* cultivars. Soybean meal had the least effect on pellet hardness, with this difference relative to the other grain varieties being more evident at the higher inclusion levels. This effect of lupin kernel meal inclusion on pellet hardness can have important practical applications through improving the durability of pellets fed using automated feeding systems. A more durable pellet has also been linked to improved nutritional outcomes with some fish species (Baeverfjord et al., 2006).

29.4.2 Effects of lupin kernel meals on water holding capacity of the extrusion mash

One of the major operating costs in feed extrusion is the depreciation of the extruder itself (Rokey, 2005a). The rate of this depreciation can be reduced significantly by increasing the fluidity of the mash being processed within the barrel of the extruder, which reduces the friction within the barrel. However, a critical moisture level is still required to be added to a mash to obtain the right rheological characteristics, to allow the feed processing to occur and induce both gelatinisation of starches and expansion of the product.

With an increasing inclusion level of the lupin kernel (*L. angustifolius*) meals and also soybean meal the feed mash had an increase in its water holding capacity (Figure 29.6). This supports that the inclusion of an increased level of carbohydrates increases the water holding capacity of the mash. This hypothesis is sustained by the observation that the effect was strongest for all *L. angustifolius* lupin kernel meal varieties and weakest for the *L. luteus* (cv. Wodjil) variety which is a strong reflection of the level of carbohydrates and particularly the inclusion of non-starch polysaccharides brought into the mash (Cheung, 1990). On a practical significance, appreciable effects were noted between the *L. angustifolius* and the reference mash, and the other grain products was observed at as little as a 10% inclusion level. With an increase in the inclusion level of each of the grain meals there was generally an increase in the water holding capacity, except with the inclusion of the *L. luteus*, which basically had no effect at all. One advantage of increased water holding capacity is the ability to extrude the diet at a lower temperature and achieve a greater degree of starch gelatinisation and decrease potential protein damage (Rokey, 2005b).

29.4.3 Effects of lupin kernel meals on RVA assessment

RVA assessment has been used as a relative, predictive tool to examine the effects of different ingredient combinations on the starch gelatinisation process (Guha et al., 1998; Sopade et al., 2006). The inclusion of *L. angustifolius* (cv. Myallie-C) kernel meal in the mash increased the rate of gelatinisation and the peak viscosity during the RVA analysis. This suggests that the inclusion of lupin kernel meals induces gelatinisation of the mash at a lower temperature than that that occurs in its absence (Sopade et al., 2006). With an increase in lupin kernel meal inclusion there was an increase in the peak viscosity, but not the rate of gelatinisation. The end viscosity at both lupin kernel meal inclusion levels was similar to that of the reference mash (Figure 29.7). A relationship can be developed between the extent of the peak viscosity and the end viscosity and the strength of pellet binding and also the bulk density of the final product. There was also significant variability in the rate of gelatinisation and peak viscosity among the different varieties of *L. angustifolius* kernel meals when included in the mash at 20% (Figure 29.8). This variability suggests that some variable component with the lupin kernel meals is affecting the temperature at which gelatinisation of the product is occurring and is probably related to the amount of water being retained by the different mixtures. All lupin varieties accelerated the gelatinisation process and also increased the peak viscosity. In some cases there was also an increase in the end viscosity as a function of the inclusion of some varieties of lupin kernel meal, such as Gungurru or W2173. These observations are consistent with the variability observed in the water retention capacity of each of the different varieties of *L. angustifolius* kernel meal (Figure 29.6).

The inclusion of *L. luteus* cv Wodjil increased the rate of gelatinisation and the peak viscosity during the RVA analysis (Figure 29.9). With an increase in *L. luteus* kernel meal inclusion there

was no increase in the peak viscosity, or the rate of gelatinisation, with a maximal effect already noted at the 10% inclusion level. The end viscosity at both inclusion levels was lower to that of the reference mash, with the greater the inclusion level resulting in lower end viscosities (Figure 29.9).

The inclusion of soybean meal reduced both the rate of gelatinisation and the peak viscosity during the RVA analysis (Figure 29.10). With an increase soybean meal inclusion there was also a reduction in the peak viscosity, the rate of gelatinisation and the end viscosity of the mash. The end viscosity at both inclusion levels was lower to that of the reference mash, with the greater the inclusion level resulting in lower end viscosities (Figure 29.10). Each of these features is consistent with a weaker bound pellet, as determined by the shear-strength test and is generally consistent with a reduction in the gelatinisation of the pellet mash in the presence of soybean meal. The lower viscosity of the soybean meal diet in the RVA assessment also suggests that there was less gelatinisation occurring overall with the use of this raw material compared to the reference diet and also the lupin kernel meal treatments. The feature of improved gelatinisation is a significant benefit to the quality of the pellets based on reports by other workers (Gleeson et al., 1999)

29.4.4 Conclusions

The findings from this study show that many of the physical features of extruded fish pellets can be modified by the inclusion of certain raw materials. The effects vary depending on the variety of raw material and also their inclusion levels. For lupin kernel meal in particular notable improvements included changes in the bulk density of the final product and the hardness of the product resulting in a more resilient pellet, suitable for automated feeding systems. The lupin kernel meals also increased the water holding capacity of the extrusion mash, which will reduce the depreciation rate of the extrusion equipment and lead to significant production savings. Use of RVA assessment showed that the inclusion of lupins influenced the starch gelling process, by both bringing it on sooner at a lower temperature and also producing a greater viscosity, which is related to a greater degree of gelatinisation. Each of these features, if managed properly and for the relevant circumstances could add significant value to extrude feeds with the inclusion of lupins based on these functional properties.

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Table 29.1. Nutrient composition of the ingredients used in the studies (all values are g/kg DM unless otherwise indicated).

Nutrient	Fish meal	Wheat	Gungarru	Merrit	Myallie-WH	Myallie - C	Mandelup	W2173	*L. luteus* cv Wodjil	Soybean meal
Dry matter content (g/kg)	931	905	920	926	927	911	910	913	913	913
Crude protein	749	142	421	429	451	427	444	466	567	531
Crude fat	87	24	80	80	74	75	75	78	67	15
Ash	161	11	25	26	28	33	27	30	39	68
Carbohydrate	0	823	473	466	446	466	454	426	327	7
Gross energy (MJ/kg DM)	20.5	18.4	20.7	20.8	20.7	20.7	21.0	21.1	21.5	18.94
Arginine	41	7	52	53	55	45	48	50	53	36
Histidine	13	1	11	11	12	11	13	12	14	9
Isoleucine	29	5	16	16	17	17	17	18	19	22
Leucine	56	10	30	31	32	30	32	33	43	39
Lysine	55	5	16	17	18	14	15	17	19	32
Methionine	21	2	4	4	4	2	4	2	5	7
Phenylalanine	30	6	18	19	20	18	18	18	21	24
Threonine	32	5	16	17	18	17	18	18	20	20
Valine	33	6	13	14	15	13	15	14	16	23

[a] Supplied by Skretting Australia, Cambridge, Tasmania, Australia. [b]Supplied by WESFEEDS Pty Ltd, Welshpool, Western Australia, Australia.

[c] Supplied by Department of Agriculture, South Perth, Western Australia, Australia. [e] Supplied by ICN Biomedical, Costa Mesa, CA, USA. [f] Supplied by Weston BioProducts, Henderson, Western Australia, Australia. [g] Supplied by Rhone Poulenc, Goodna, Queensland, Australia. [h] Supplied by SIGMA, St Louis, Missouri, United States.

Table 29.2. Formulations of the extrusion mash diets (all values are g/kg).

Diet Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
Fishmeal	700	630	630	630	630	630	630	630	630	560	560	560	560	560	560	560	560	490	–	490	490	–	–	490	490
Soybean meal	0	100	0	0	0	0	0	0	0	200	0	0	0	0	0	0	0	300	–	0	0	–	–	0	0
L. angustifolius cv Gungarru	0	0	100	0	0	0	0	0	0	0	200	0	0	0	0	0	0	0	–	0	0	–	–	0	0
L. angustifolius cv Mandelup	0	0	0	100	0	0	0	0	0	0	0	200	0	0	0	0	0	0	–	300	0	–	–	0	0
L. angustifolius cv W2173	0	0	0	0	100	0	0	0	0	0	0	0	200	0	0	0	0	0	–	0	300	–	–	0	0
L. angustifolius cv Myallie-WH	0	0	0	0	0	100	0	0	0	0	0	0	0	200	0	0	0	0	–	0	0	–	–	0	0
L. angustifolius cv Merrit	0	0	0	0	0	0	100	0	0	0	0	0	0	0	200	0	0	0	–	0	0	–	–	0	0
L. angustifolius cv Myallie-REF	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	200	0	0	–	0	0	–	–	300	0
L. luteus cv. Wodjil	0	0	0	0	0	0	0	0	100	0	0	0	0	0	0	0	200	0	–	0	0	–	–	0	300
Wheat	144	130	130	130	130	130	130	130	130	115	115	115	115	115	115	115	115	101	–	101	101	–	–	101	101
Vitamin and mineral premix	5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4.5	4	4	4	4	4	4	4	4	3.5	–	3.8	3.5	–	–	3.5	3.5
Yttrium oxide	1	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	–	0.7	0.7	–	–	0.7	0.7

Table 29.3. Physical properties of all extruded diets.

	Basal	**Soybean meal**			**Myallie - C**			**Myallie - WH**			**Mandelup**		
	0%	**10%**	**20%**	**30%**	**10%**	**20%**	**30%**	**10%**	**20%**	**30%**	**10%**	**20%**	**30%**
Bulk density (g/L)	531	560	618	556	546	626	653	603	652	–	597	656	674
Vacuum oil uptake (g/kg)	560	411	333	370	384	301	240	323	288	–	351	318	247
Radial expansion (%)	24%	17%	11%	20%	13%	9%	12%	12%	9%	–	13%	9%	13%
Sink rate (cm/s)	0.0	0.0	-4.4	-4.6	0.0	-4.2	-6.7	-1.3	-5.9	–	-2.4	-6.4	-
Shear strength (g)	406	486	572	621	538	668	776	502	574.57143	–	504	576	668
	Basal	Merrit			Gungurru			W2173			*L. luteus* cv. Wodjil		
	0%	10%	20%	30%	10%	20%	30%	10%	20%	30%	10%	20%	30%
Bulk density (g/L)	531	599	658	–	599	648	–	576	648	671	623	694	664
Vacuum oil uptake (g/kg)	560	292	276	–	325	273	–	378	333	256	485	338	240
Radial expansion (%)	24%	12%	9%	–	15%	9%	–	13%	9%	13%	11%	3%	12%
Sink rate (cm/s)	0	-1.5	-6.3	–	-0.4	-5.6	–	0.0	-8.3	0.0	-4.8	-7.5	-6.4
Shear strength (g)	406	499	617	–	503	520	–	443	577	655	523	585	684

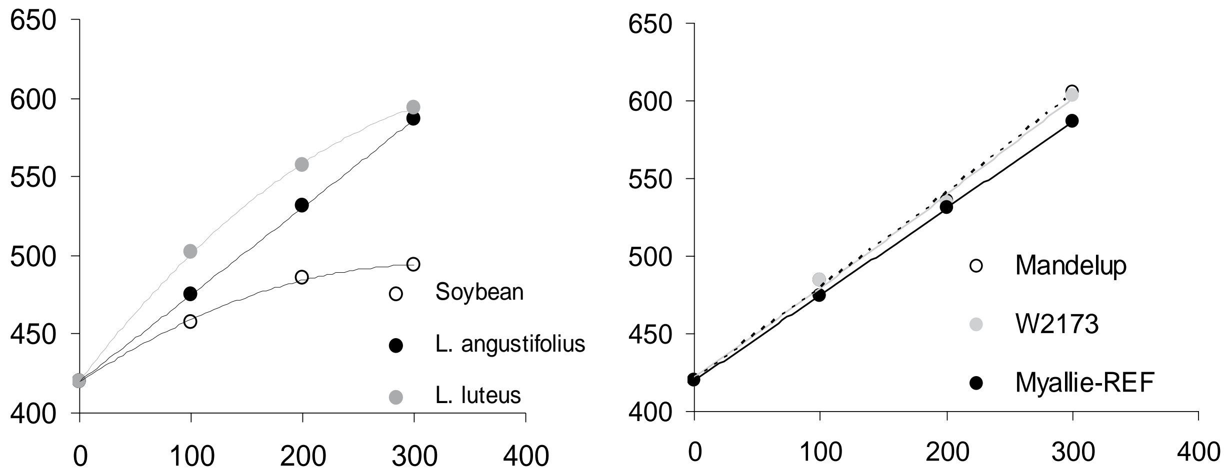

Figure 29.1 Pellet bulk density (g/L) as a function of grain variety and inclusion level. (A) the influence of grain species, (B) the influence of grain cultivar of *L. angustifolius***.** The *L. angustifolius* is the Myallie-REF variety in both cases.

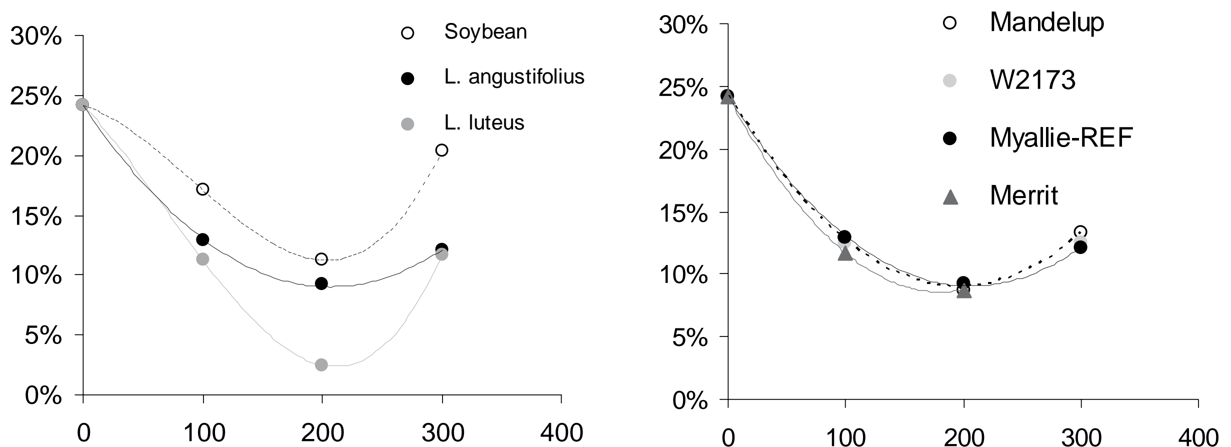

Figure 29.2 Pellet expansion (%) as a function of grain variety and inclusion level. (A) the influence of grain species, (B) the influence of grain cultivar of *L. angustifolius*. The *L. angustifolius* is the Myallie-REF variety in both cases.

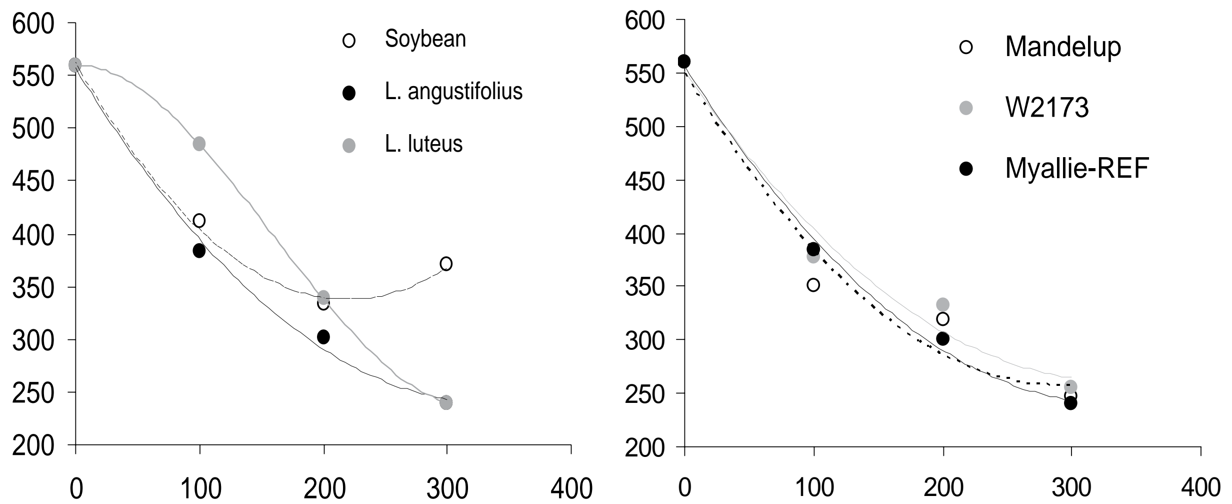

Figure 29.3 Pellet vacuum oil uptake (g/kg) as a function of grain variety and inclusion level. (A) the influence of grain species, (B) the influence of grain cultivar of *L. angustifolius*. The *L. angustifolius* is the Myallie-REF variety in both cases.

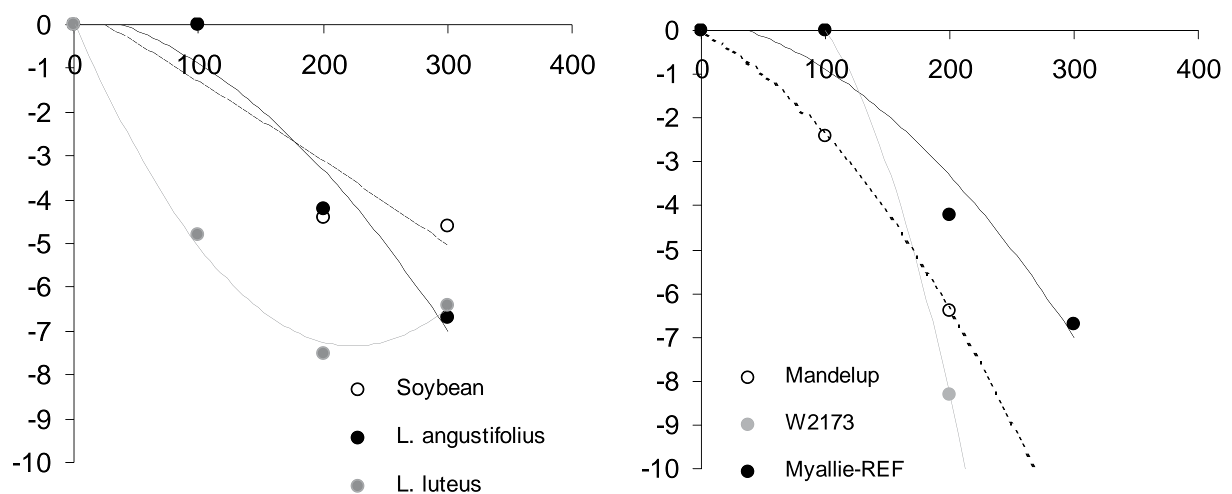

Figure 29.4 Pellet sink rate (cm/s) as a function of grain variety and inclusion level. (A) the influence of grain species, (B) the influence of grain cultivar of *L. angustifolius*. The *L. angustifolius* is the Myallie-REF variety in both cases.

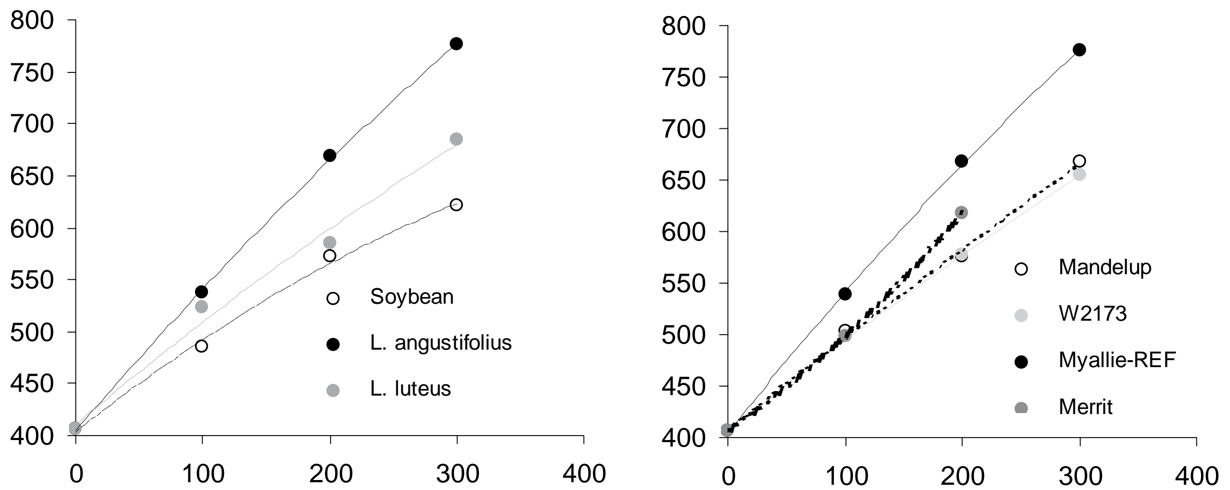

Figure 29.5 Pellet hardness (g of force to split) as a function of grain variety and inclusion level. (A) the influence of grain species, (B) the influence of grain cultivar of *L. angustifolius*. The *L. angustifolius* is the Myallie-REF variety in both cases.

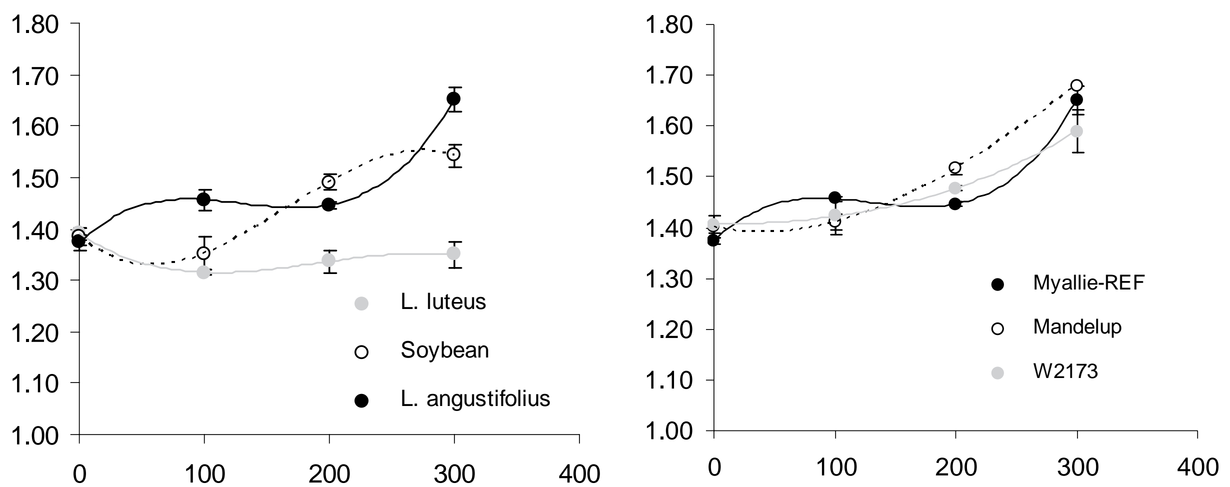

Figure 29.6 Water retention as a function of grain variety and inclusion level. (A) the influence of grain species, (B) the influence of grain cultivar of *L. angustifolius*. The *L. angustifolius* is the Myallie-REF variety in both cases.

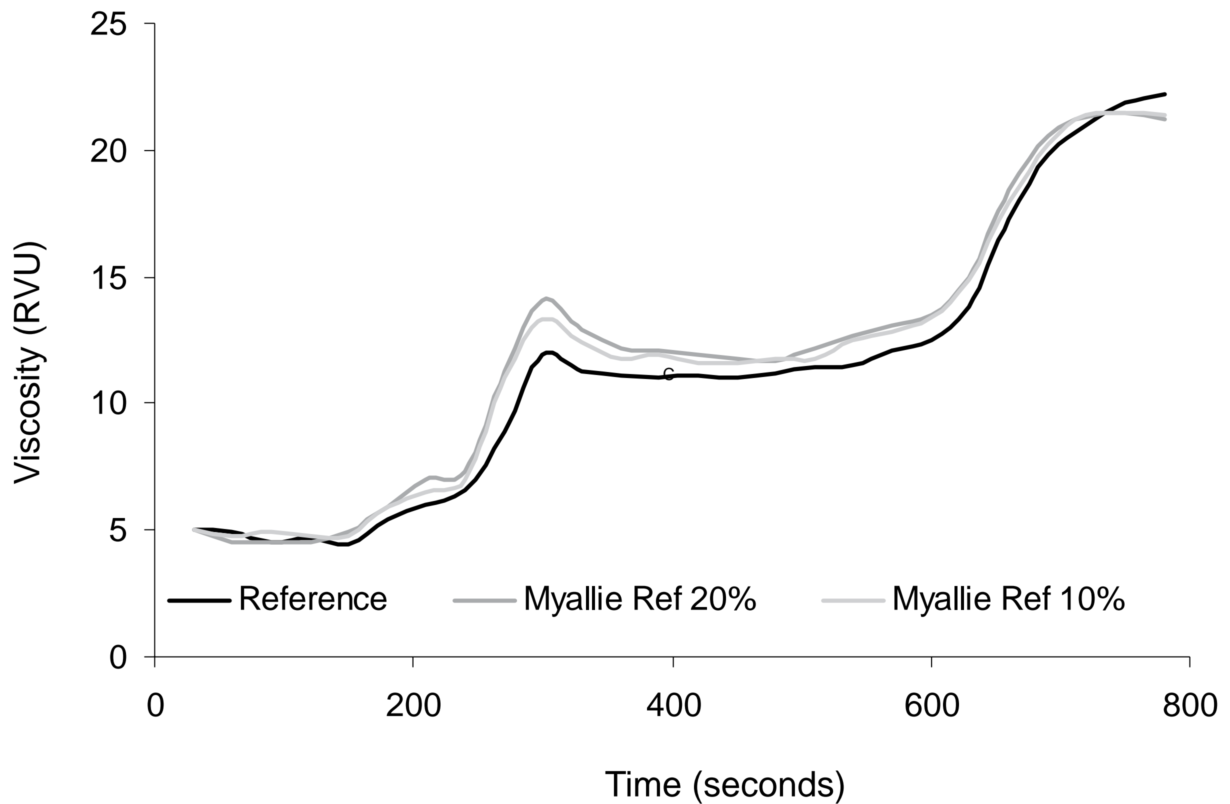

Figure 29.7 RVA profiles of Reference, Myallie-REF 10% and Myallie-REF 20% mashes using standard heating profile 1.

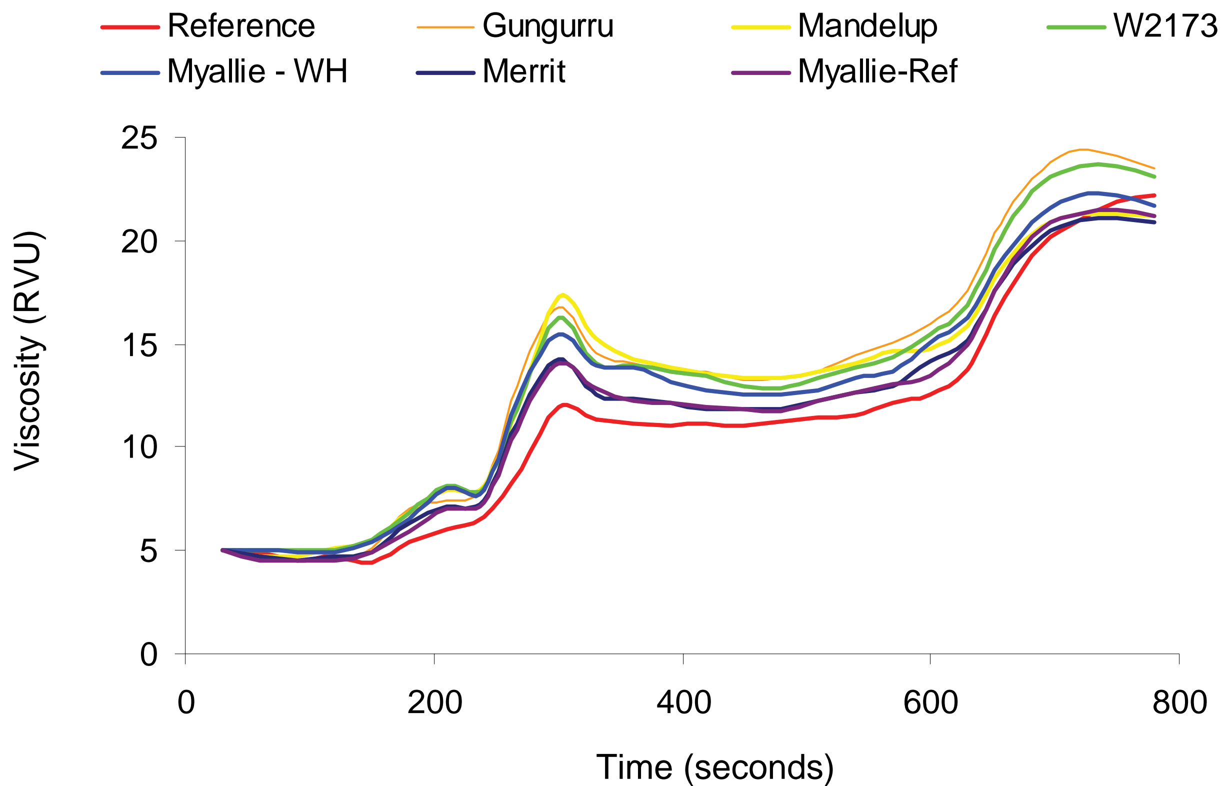

Figure 29.8 RVA profiles of Reference and all *L. angustifolius* kernel meal 20% inclusion mashes using standard heating profile 1.

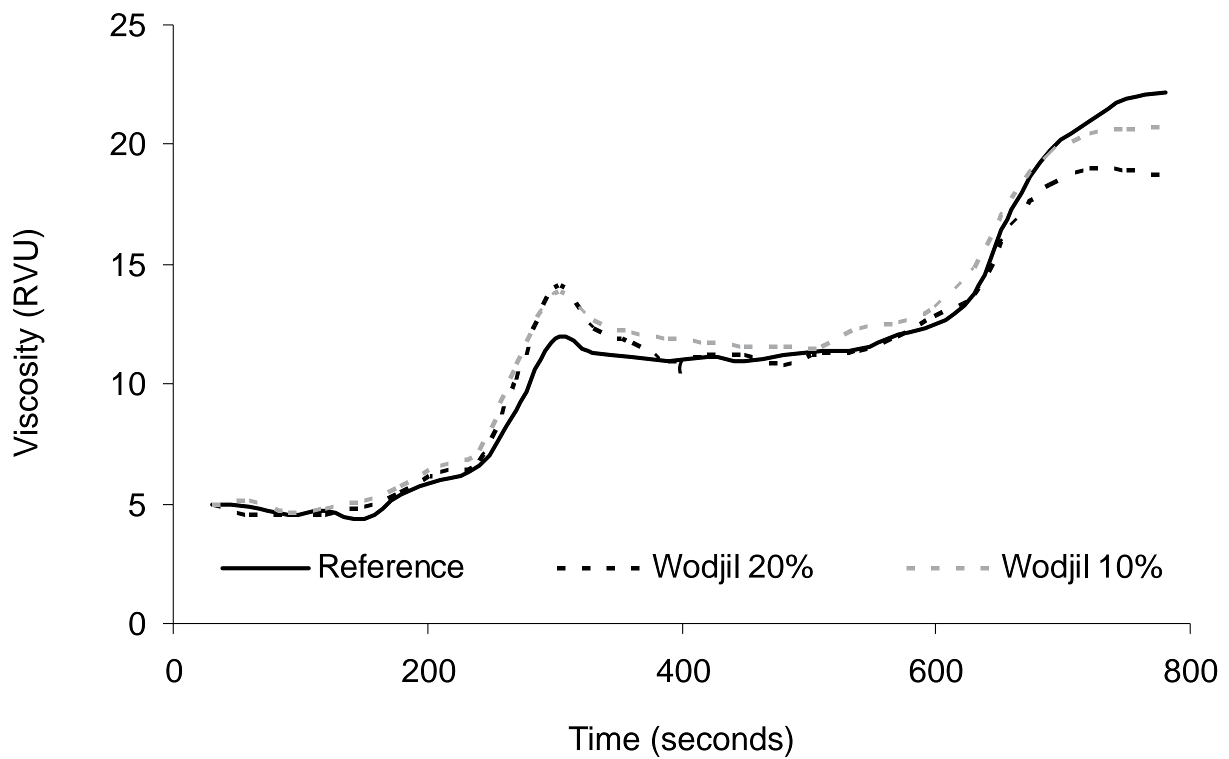

Figure 29.9 RVA profiles of Reference and *L. luteus* kernel meal at 10% and 20% inclusion mashes using standard heating profile 1.

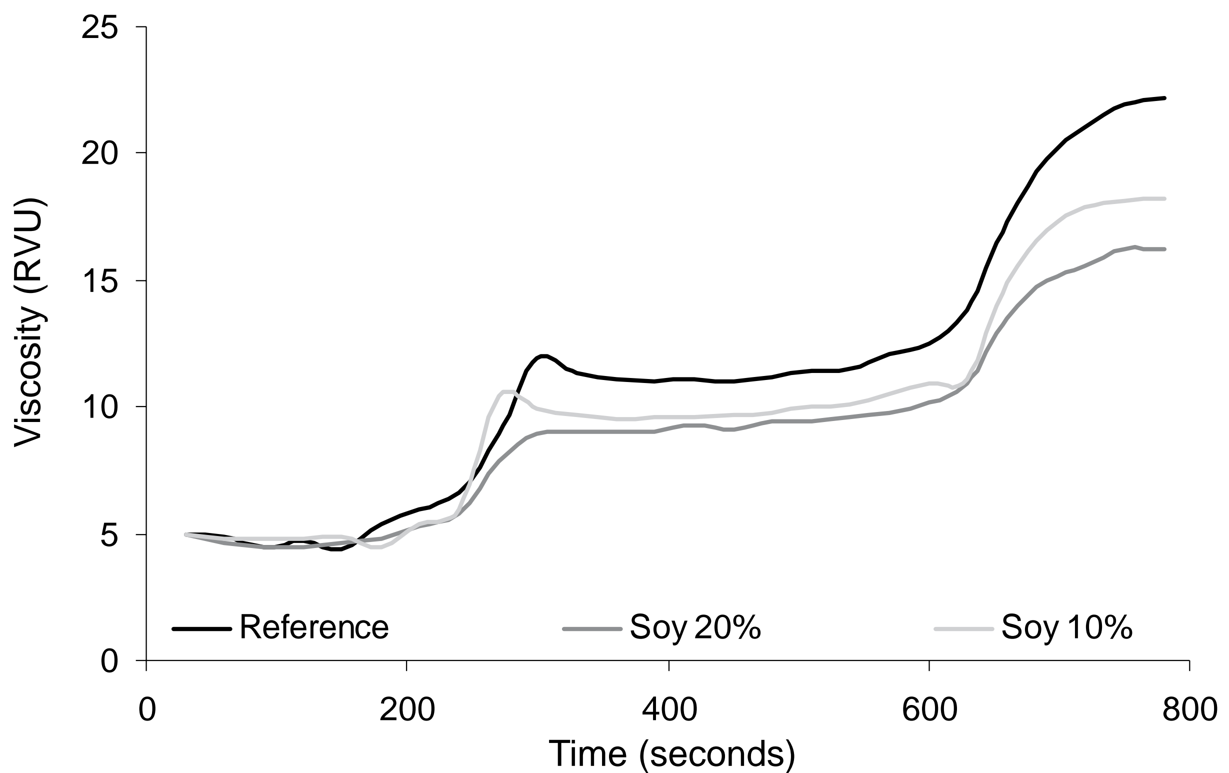

Figure 29.10 RVA profiles of viscosity (RVU) of the Reference and Soybean meal at 10% and 20% inclusion levels using standard heating profile 1.

30.0 Technology extension, evaluation of commercially supplied value-added grain products and uptake by industry of research outcomes

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Abstract

Planning and technology dissemination workshops were held in 2003, 2004, 2005 and 2007. At these workshops, key industry personnel were engaged to provide input to the research planning process and also to pass on key elements of knowledge gained from the research conducted to that point. A proceeding was published from each workshop, which was used to further promote the work. A series of industry collaborative studies were also undertaken to examine the composition, digestibility and palatability to rainbow trout of different types of value-added grain products. Details of each product and their assessment were conducted on a commercial-in-confidence basis and as such no details will be provided. A total of eight products from each company were evaluated over a two-year period. In addition to the evaluation of the commercially supplied value-added grain products, a large (n=75) sample set of lupin meals was collected, prepared, analysed and evaluated for their digestible energy and nutrient values. This data was then supplied to each of the collaborating commercial partners, along with samples of the kernel meal, to allow the development of calibrations for chemical and nutritional properties using near infrared spectroscopy (NIRS). Notably, each collaborator had different NIRS equipment and accordingly required their own sample set to allow the development of calibrations. Visits were conducted to feed companies in Norway, Scotland, Japan, Thailand and Chile to promote the potential for lupin use in aquaculture feeds were undertaken in 2004, 2005 and 2006. Significant uptake of the use of lupin kernel meals by major international feed companies, like Skretting Australia has occurred since 2002. From uptake by this company, use of the raw material has been broadly adopted throughout the companies international operations in Norway, Chile and Japan. To take advantage of a growing international market for lupin kernel meals a joint-venture company Australasian Lupin Processing Pty Ltd was initiated to establish the world's largest lupin dehulling plant in Forrestfield, Western Australia. Supply of kernel meals to the aquaculture market has been touted as one of the major sectors underpinning the development of this initiative.

30.1 Introduction

Part of the objective of this program was to instigate the industrial adoption of value-added lupin products in feeds for the aquaculture sector. To achieve this a significant extension component was undertaken to provide technical outputs and services to stakeholders using a process of workshops, promotional site visits and collaborative trials. The inclusion of key partners of CBH-Group, Weston Technologies (George Weston Foods) and Skretting Australia, who all contributed significant financial inputs to the program, was instrumental in this extension process. The processes and a summary of key outputs are summarised in this chapter.

30.2 Extension

Throughout the life of the Aquaculture Feed Grains Program a series of workshops were held in Fremantle, Western Australia in 2003, 2004, 2005 and 2007. The workshops served a dual purpose of providing extension of the knowledge gained to each point in time and also seeking input from select stakeholders. The workshops were conducted on an invitation only basis to ensure the optimum group size and optimise synergies between stakeholders. A proceeding was produced from each workshop that has been published as further used as extension material (Figures 30.1, 30.2, 30.3 and 30.4).

In 2004, 2005 and 2006 site visits were made to key aquaculture feed companies in Australia, Norway, Chile, Scotland, Japan and Thailand to promote the use of lupin kernel meals and the work of the Aquaculture Feed Grains Program. From these visits the feed companies made numerous enquiries to grain suppliers and in some cases trial shipments from 100 kg to 20,000 kg were instigated.

30.3 Industry adoption

For commercial-in-confidence reasons it is not possible to divulge the relative uptake of lupin kernel meals by each country, other than by 2005 about 20,000 to 30,000 tonnes per annum of Australian origin lupin kernel meal was being used in aquaculture feeds throughout the world. Chilean industries have begun adopting use of locally (Chilean) grown lupins and reports of volumes vary between 10,000 and 40,000 tonnes per annum in 2006. The drought of 2006 has caused significant problems for continuity of supply of lupin products and significant promotional work will probably be required to re-instigate the trade process once normal production of lupins is reinstated.

The inclusion of key partners of CBH-Group, Weston Technologies and Skretting Australia in the program has helped facilitate the uptake of knowledge and industrial adoption of lupin kernel meal production and use in aquaculture feeds (see Figures 30.5, 30.6, 30.7, 30.8 and 30.9). This industrial adoption has resulted in significant volumes of lupin kernel meal being used by the Skretting group, both domestically and internationally (Figure 30.7). In response to this emerging demand for value-added lupins, CBH-Group and Weston Technologies formed a joint venture to established Australasian Lupin Processing Pty Ltd (Figure 30.8). The new company Australasian Lupin Processing Pty Ltd began full-scale commercial production in early 2007 (Figure 30.9).

As part of the programs activities evaluations of commercial products produced by both CBH-Group and Weston Technologies were undertaken in 2004 and 2005. These evaluations remain commercial-in-confidence.

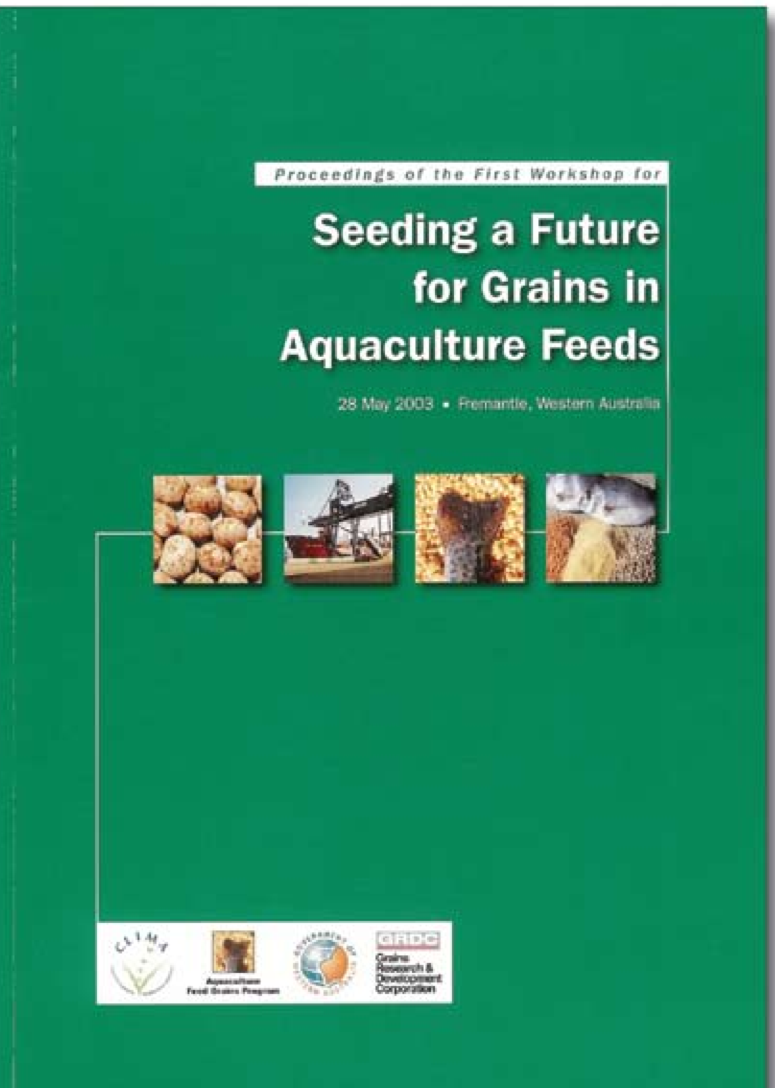

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Figure 30.1 Cover and Contents of the Proceedings of the First Workshop for “Seeding a Future for Grains in Aquaculture Feeds – 28th May 2003”.

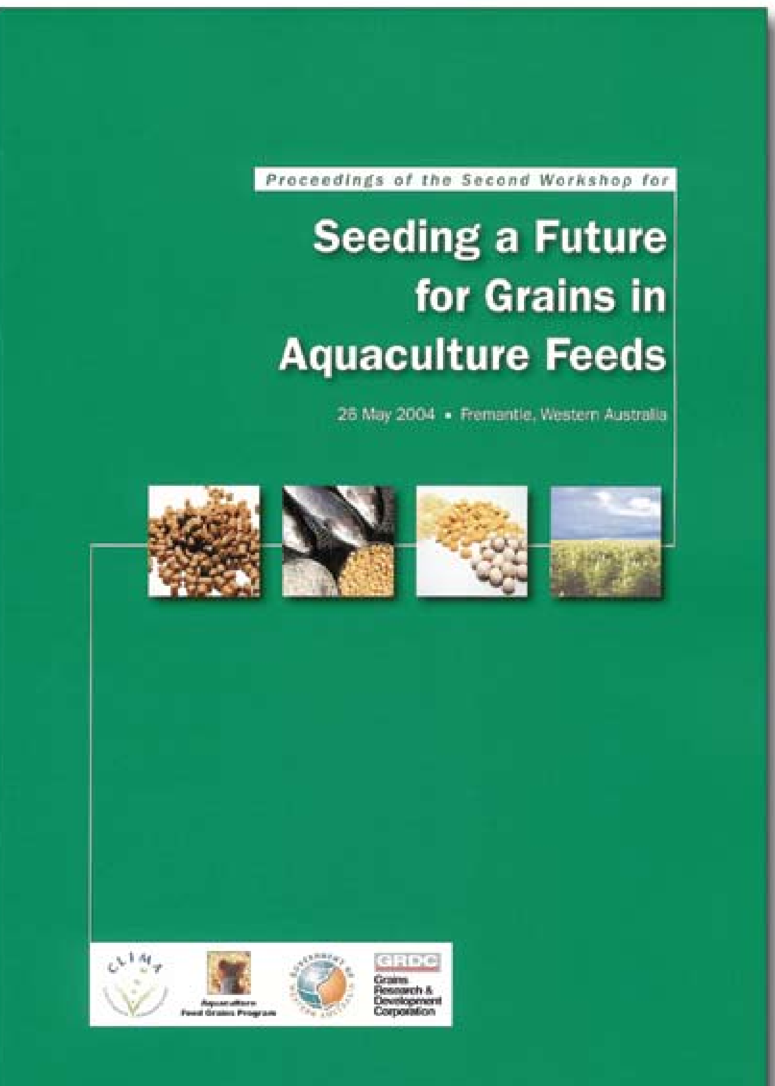

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Figure 30.2 Cover and Contents of the Proceedings of the Second Workshop for “Seeding a Future for Grains in Aquaculture Feeds – 26th May 2004”.

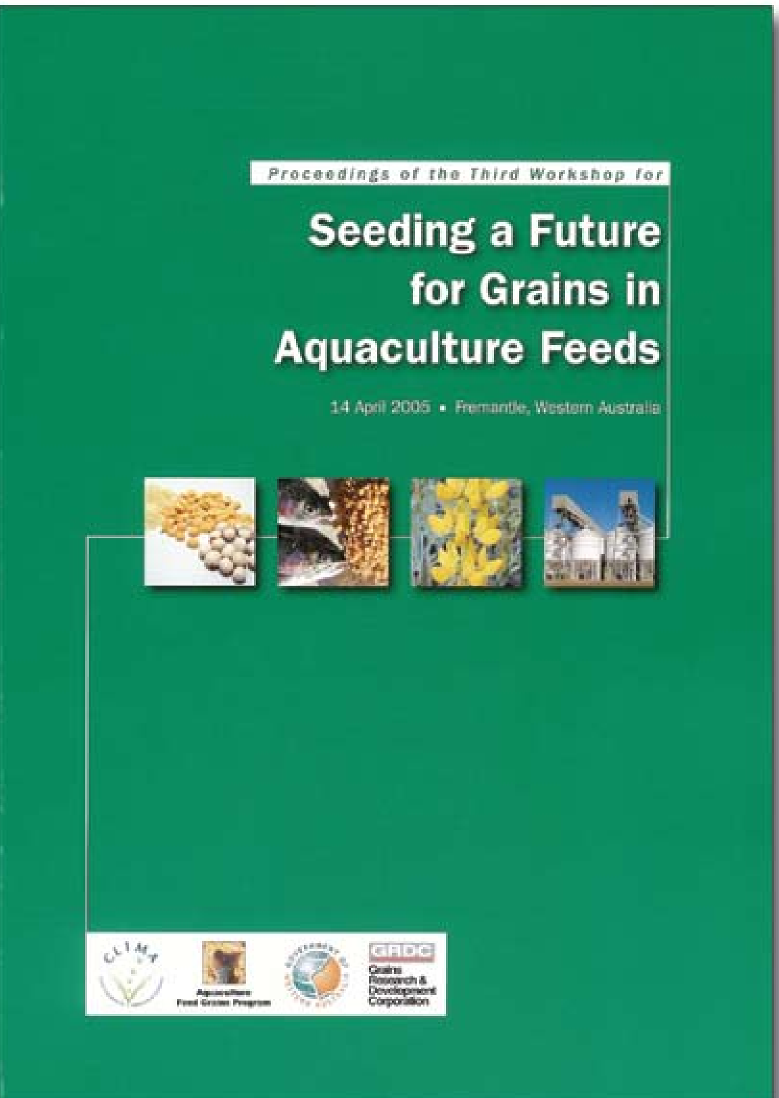

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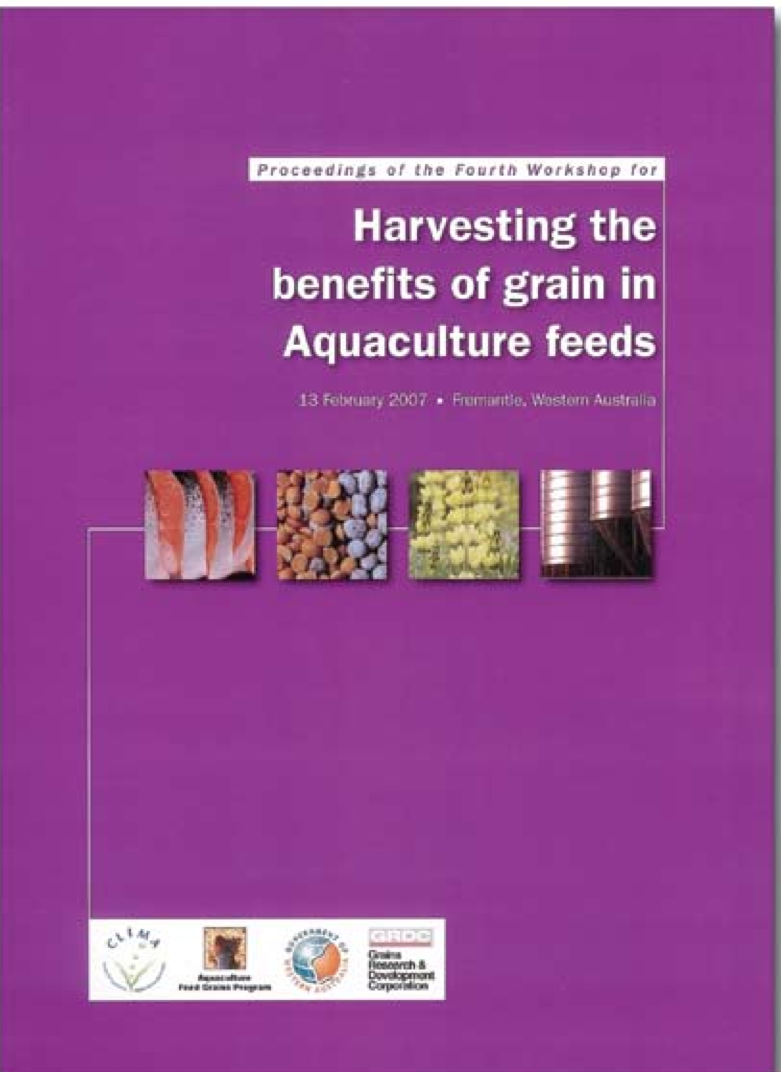
Figure 30.3 Cover and Contents of the Proceedings of the Third Workshop for "Seeding a Future for Grains in Aquaculture Feeds – 14th April 2005".



Proceedings of the Fourth Workshop for

Harvesting the benefits of grain in Aquaculture feeds

13 February 2007 • Fremantle, Western Australia

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Figure 30.4 Cover and Contents of the Proceedings of the Fourth Workshop for "Harvesting the Benefits of Grains in Aquaculture Feeds – 13th February 2007".

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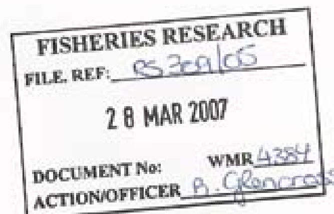

22 March 2007

Dr Brett Glencross
Principal Research Scientist
Research Division
Department of Fisheries
PO Box 20
NORTH BEACH WA 6920

Dear Brett

CBH Group, as a partner of the Aquaculture Feed Grains Program, acknowledges the Program's support in assisting its research into understanding the value of lupins and lupin fractions in aquaculture feeds.

This work has enabled us to evaluate a series of CBH developed products over several years. As well, the work has included the coordination and facilitation of the development of data and samples for NIR calibration curves.

The professional relationship between CBH, principally through Mark Tucek, and yourself has been consolidated over a number of years through consultation on project directions, feedback on the progress of the projects, facilitation of industry networks and support in overseas missions to key S-E Asian customers.

Given the focus of my Department on CBH customer quality requirements, we are vitally aware of the limitations of our environment for protein achievement and recognise the important role that lupins play in addressing that issue. The work that has been undertaken within the Aquaculture Feed Grains Program has been valuable both for better understanding the role of lupins within aquaculture and in fostering industry collaboration. It has had a strategic role in exploring new higher

Figure 30.5a Letter from Cooperative Bulk Handling Pty Ltd.

value options for lupins in the market place that, if successful, will strengthen their place in crop rotations to the overall benefit of the cropping industry.

Yours sincerely

For: Co-operative Bulk Handling Limited

Peter Portmann
Manager Technical Market Services

Figure 30.5b Letter from Cooperative Bulk Handling Pty Ltd.

FISHERIES RESEARCH
FILE. REF: RS309/05
19 APR 2007
DOCUMENT No: WMR 4410
ACTION/OFFICER B. Glencross

23rd March 2007

Dear Brett

As a partner of the Aquaculture Feed Grains Program, George Weston Technologies gratefully acknowledges the program's support in assisting its research in understanding the value of lupins and lupin fractions in aquaculture feeds and the promotion of their use.

This work has centred on the evaluation of a series of George Weston Technologies products over several years. As well, the work has included the coordination and facilitation of the development of data and samples for Near InfraRed Spectrometry (NIRS) calibration curves.

The professional relationship between George Weston Technologies and yourself has been consolidated over a number of years. This has involved consultation on project directions, timely feedback on the progress of the projects and facilitation of the development of industry networks.

GWT acknowledges the value of the work that has been done so far by the Aquaculture Feed Grains Program and are keen to ensure that the exchange of information among industry professionals is maintained.

Your's sincerely

Chief Executive Officer
George Weston Technologies
Peter Schutz

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A division of George Weston Foods Limited ABN 45 008 429 632

Figure 30.6 Letter from Weston Technologies Pty Ltd.

Dr Brett Glencross
Principal Research Scientist
Department of Fisheries
PO Box 20
North Beach WA 6920

14th June 2007

Dear Brett,

I am writing to you on behalf of Skretting to thank you for the opportunity to join the Aquaculture Feed Grains Program, which over recent years has advanced the use of lupin meals in finfish feed worldwide.

In close collaboration with Skretting Australia, the program furthered the understanding of the nutritive suitability of lupin products for many of the commercially significant global aquaculture species. Refinement of near infrared (NIR) spectra for lupin kernel meal has been a key achievement in this collaboration. It has advanced Skretting's capability to rapidly measurement nutritive content and has delivered a significant improvement to our quality assurance program. The program also appropriately evaluated the physical characteristics of lupin meals, which translated to further understanding of process requirements in commercial finfish feed manufacture.

Skretting appreciate the professional relationship we have with you. Importantly, this relationship has extended further than the local Skretting operation, and your collaboration with many of the international Skretting operations and Skretting Aquaculture Research Centre (ARC) has been a credit to your professional approach. It has ensured the Aquaculture Feed Grains Program has achieved international commercial recognition.

Your work has been a significant driver of lupin usage in finfish feeds, which now sees lupin kernel meal used in all Skretting operations around the world when economically viable.

Again, Skretting thanks you for the opportunity to collaborate with the Aquaculture Feed Grains Program over recent years, and we look forward to participating in further work between yourself and Skretting in the future.

Yours Sincerely,

James Rose
Managing Director
Skretting Australia

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a nutreco company

Figure 30.7 Letter from Skretting Australia Pty Ltd.

>>>>>MEDIA RELEASE MEDIA RELEASE MEDIA RE

First load of de-hulled lupins makes its way to market

The first load of de-hulled lupins from the Australasian Lupin Processing (ALP) plant in Forrestfield has made its way into the domestic market, following the recent first sale of the product by Grain Pool to Milne Agrigroup.

ALP was established as a joint venture between the CBH Group and George Weston Foods in 2004 to develop a lupin de-hulling plant in Western Australia that will be the first of its kind to provide an enhanced quality of lupin kernels to the market in bulk quantities.

The first load of de-hulled lupin kernels left the plant last Wednesday, 17 January 2007 and will be used for stock feed.

John Doncon, Australasian Lupin Processing Plant Manager said that while the plant is still to be officially commissioned for operation, the test samples indicated the lupins were of high enough quality to start selling into the market.

"The original test results were very promising and clearly indicated to us that the product was good enough to sell into the market," Mr Doncon said.

"The results from the first load of de-hulled lupins produced protein levels at around 40%. Prior to processing, the lupins were recording protein at only around 32%.

"The initial reports from Milne Agrigroup following the delivery of the first load last week have been extremely positive and they have been particularly happy with the quality of the de-hulled lupins from the plant."

Mr Doncon said the finishing touches to the plant are now being made, with the mill anticipated to be fully commissioned in the first quarter of 2007.

"The plant will have a total capacity of 200,000 tonnes and will be the largest of its kind in the world," he said.

"We expect to be able to process around 20,000 tonnes of 2006-07 season lupins this year and continually increase on the tonnages processed per annum following.

"Western Australia is a significant origin of high-quality lupins. Processing this grain to augment its nutritional value will increase its appeal to domestic and international markets and the lupin de-hulling plant will allow the growers of Western Australia to increase their links to both domestic and international end-user customers.

Figure 30.8 Media release from CBH promoting the establishment of Australasian Lupin Processing Pty Ltd.

Australasian Lupin Processing Plant
700 Abernethy Road
Forrestfield WA 6058
Tel: 9454 0306 Fax: 9454 9008

MEDIA RELEASE

Australasian Lupin Processing now open for business

Australasian Lupin Processing's lupin de-hulling plant in Forrestfield is now officially open for business, following full commissioning of the facility in May 2007.

Australasian Lupin Processing was established as a joint venture between the CBH Group and George Weston Foods in 2004 to develop a lupin de-hulling plant in Western Australia that is the first of its kind to provide an enhanced quality of lupin kernels to the market in bulk quantities.

John Doncon, Australasian Lupin Processing Plant Manager said the plant has been producing test samples for market since January of this year and has produced around 7,000 tonnes of de-hulled lupins to date.

"We are pleased to now be fully operational and anticipate production of around 20,000 tonnes of 2006-07 season lupins, with an increase in production to around 60,000-70,000 tonnes of 2007-08 season lupins, depending on how the season pans out," Mr Doncon said.

"The plant has a total capacity of 200,000 tonnes and is the largest of its kind in the world, incorporating brand new technology and processes to ensure the quality needs of customers are effectively met."

Rod Sands, Chairman of Australasian Lupin Processing said the development of the lupin de-hulling plant in Forrestfield provides a unique opportunity to create additional value for Western Australian grain growers.

"The processing of high quality lupins from Western Australia to augment their nutritional value allows us to create new markets for WA lupins and allows growers to secure greater returns for their core product," Mr Sands said.

"For example, lupin kernels are a high protein product and have exciting applications in the rapidly expanding aquaculture market.

"With world fish stocks declining, the aquaculture market is looking for alternative vegetable protein sources to replace traditional fish protein sources and lupin kernels are well placed to fill this need.

"We look forward to our first full year of operation and in providing WA growers with access to new markets and added value for their raw product."

Dated: 7 June 2007

Media Contact: Nicole Penter
Media Advisor, CBH Group
Ph: (08) 9237 9712 Mob: 0427 087 123

Figure 30.9 Media release from Australasian Lupin Processing Pty Ltd promoting the commencement of commercial value-adding of lupins.

31.0 General Discussion – Harvesting the Benefits of Grains in Aquaculture Feeds

Brett Glencross[1,2]

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[2] Centre for Legumes in Mediterranean Agriculture (CLIMA) - Aquaculture Feed Grains Program, University of Western Australia, Crawley, WA 6909, Australia.

Abstract

A series of projects were undertaken to develop both the potential of lupins as a feed grain for the aquaculture feed sector, and to also facilitate the adoption of this grain by this feed sector. The objectives of these activities were to create a new, higher-value market for lupins based on a local, value-adding industry and to reduce resource risk for the aquaculture industry by reducing their reliance on fish meal as a protein source. A range of value-added grain products were developed from several grain varieties and the methods for their production detailed and transferred to industry. At the instruction on the industry partners a focus was made on the development and assessment of lupin kernel meals. It was demonstrated that the use of lupin kernel meals adds significant value to the seed equivalent price of lupins. Through the course of the program major advances on the understanding of grain application to aquaculture feeds have also been made. These include the demonstration that grain protein can be utilized by fish as efficiently as that of fish meal protein, the determination of key compositional factors that influence the digestible protein and energy value of lupin kernel meals to fish and the development of the worlds first near infrared spectroscopy calibration for assessment of digestible and crude composition parameters from a feed grain for use in fish diets. As an outcome of this work lupin kernel meals have gained widespread acceptance and adoption throughout the Australian and international aquaculture feed industry. Significant volumes of this value-added grain are now being used in Australian aquaculture feeds thereby reducing reliance on fish meal as a protein source. A major industrial grain value-adding facility has also been commissioned with the intent of producing lupin kernel meals, with the aquaculture feed sector identified as their key market.

31.1 Introduction

Like all research programs, the end-point is rarely black-and-white. Progress achieved in certain areas, highlights deficiencies in others, new findings in one aspect point to new leads in another. The research presented in this report was done with the intent of improving our understanding of the nutritional characteristics of a range of grain resources, but with a specific focus on lupins and their potential for aquaculture feeds. The primary objective of this work was to improve our ability to use these resources in aquaculture diets in both nutritional and functional contexts, thereby improving the market potential for the feed grains and also increasing the confidence of the feed sector in using these raw materials.

Many of the outcomes achieved from this research have already strengthened the position of grain products in general and lupins in particular, as ingredients to be used by the aquaculture feed industry. The outcomes have also served the grain processing sectors interests by clearly defining some of the quality criteria that will be important to the aquaculture sector, which has established itself as a premium-paying sector in the feed grain market.

31.2 Grains and Value-adding

A range of value-added lupin products were developed, refined and evaluated in this program. Lupin kernel meals consistently proved to be one of the more viable products to produce though a process of dehulling and air-aspriation to remove the hulls. The dehulling of lupins was shown to significantly improve their nutritional value to fish (Chapter 4). Notably there was a linear increase in digestible energy value observed, while a curvilinear response in digestible protein value was observed. This finding shows that there is not only significant improvements in the protein content of the value-added product, but that there are also nutritional benefits to the fish in using these value-added products over whole lupins. These findings are consistent with other studies that also show that there are both compositional and nutritonal benefits from dehulling lupins (Petterson, 2000; Booth et al., 2004).

The kernel meals from both *L. angustifolius* and *L. luteus* were evalauted in several aquaculture species in this program (Chapters 5, 14, 21, 24 and Chapter 25). Kernel meals of *L. luteus* had significantly higher levels of digestible protein and energy than *L. angustifolius* kernel meals in virtually every case. This vareity of value-added grain also provides improved potential for the replacment of a greater proportion of fish meal used in aquaculture diets, because of its higher protein and energy levels. Indeed, the composition specifications of *L. luteus* kernel meal are close to those identified for an ideal value-added grain product for use in aquaculture feeds (Chapter 12). These findings were consitent with earlier reports (Glencross and Hawkins, 2004; Glencross et al., 2004).

A variety of protein concentration methods were examined where either dry or wet processing options were considered. The dry methods were observed to be more effective in increasing the protein content (30% to 41%), but had poor yield efficiencies. The initial wet extraction methods had lower relative increases in protein (55% to 59%), but had significantly better yields. A key part of this preliminary process of concentrate development was the linear-least cost modelling of different hypothetical product options. Modelling of a hypothetical grain protein concentrate use suggested that a product with a protein level in the range of 50% to 60% would be optimal for use in salmonid feeds and provide the most likely economic feasibility and greatest level of replacement of fishmeal. This identification of an "ideal" protein level is consistent with the actual protein content of several other commercially produced protein concentrates (Refstie et al., 1998). Ironically, the composition of kernel meals from both *L. luteus* and *L. mutabilis* are also within this "ideal" range.

Further wet extraction protein concentration methods were examined based on protein isolation technologies adopted from the soybean industry (Lasztity et al., 2001). Using both protein concentration and isolation techniques, a series of protein enriched products were prepared from *L. angustifolius*, *L. luteus* and *L. mutabilis* kernel meals (Chapter 15). Using protein isolation methods it was possible to produce products with protein levels in excess of 80% (Chapter 13). Protein concentration methods produced products of a lower protein content, but had a greater yield (Chapters 12 and 13). Both yield and and protein content will be important factors in determining the commercial viability of the final products. Each of the prototype protein concentrates made from *L. angustifolius* and *L. luteus* kernel meals were highly palatable and digestible when fed to either rainbow trout or Atlantic salmon (Chapter 19). The drying process was also identified as a key cost-viability factor.

Several different drying methods were examined in the production of protein concentrates to consider the implications of difference processes on the product quality (Chapter 17). While

freeze-drying proved to be a useful experimental/laboratory scale method that produced a light, low-density, friable powder, it was not considered a viable industrial scale method. For up-scaling, spray-drying and ring-drying technologies were examined with both *L. angustifolius* and *L. luteus* protein isolates. Spray-drying proved to produce a good, consistent product. Ring-drying was not viable and was observed to gum the products and not produce a useful product.

31.3 Nutritional quality

The nutritional quality of a raw material for feed is generally regarded as the comparitive ability of that raw material to supply nutrients to the animal to which it is being fed (van Barneveld, 2001). Part of this quality assessment is based on the overall composition of the raw material. Other important factors affecting the nutritional quality of a feed grain include the digestibility of the nutrients and energy, and also the type and concentration of anti-nutritional factors and the capacity of the animal to utilise the digested nutrients for growth (Francis et al., 2001; Glencross et al., 2007). The general raw material evaluation strategy used in this program was based on that detailed in Glencross et al. (2007).

31.3.1 Lupin kernel meal quality

31.3.1.1 Digestibility

Substantial variability in the kernel meal composition of *L. angustifolius* was noted across the combined studies in this program. Across a collection of 75 different samples a (mean ± S.D.) protein level of 45.4 ± 3.45% on a dry basis was determined. Protein levels in the kernel meals varied from 36.5% and 56.7%. Limited variability was observed in the lipid or ash content of the kernel meals, so any variance in the protein levels was usually offset by a change in the level of carbohydrate. Most carbohydrates in lupins are non-starch polysaccahrides (Petterson, 2000). A series of the kernel meals that were produced from seed collected from three successive years production of commercial cultivars grown that the same site showed substantial variability in composition. In these samples the effect of year on composition more pronounced that that of cultivar.

The determination of the ability of an animal to absorb nutrients from a raw material is another attribute important to raw material quality assessment. This is usually assessed by determining the comparative and absolute digestibiltiies of key nutrients and energy from diets in which the raw material have been included (Chapters 5, 7, 11, 14, 21, 22, 24 and 25). A comparison of different digestibility assessment methods showed that high levels of carbohydrate in the diet resulted in greater disparity between the results observed with the different methods (Chapter 14). Faecal stripping methods consistently provided more conservative estimates of the digestibility parameters for fins-fish but are not appropriate for use in studies with prawns.

The influence of lupin kernel meals, soybean meal and a lupin protein concentrate on gut transit in Atlantic salmon was also examined using a marker replacement method. The results of this work showed that the inclusion of lupin kernel meals increased the rate of gut transit of the feed compared to the effects induced by the inclusion of soybean meal or a lupin protein concentrate.

Substantial variability in the digestibility of dry matter (39.1% to 65.5%), protein (65.5% to 114.6%), amino acids (52.0% to 126.5%) and energy (48.2% to 69.4%) was observed from

lupin kernel meals fed to either rainbow trout or Atlantic salmon (Chapters 7, 14, 19, 20, 22, 24). Variability in the digestible protein and energy value of the lupin kernel meals when fed to rainbow trout was shown to be related to kernel meal composition (Chapter 7). Higher protein levels in the meal correlated with better protein and energy digestibility. The high protein levels also correlated with lower non-starch polysaccharide (NSP) levels in the kernel meals and this resulted in a concommitant relationship between protein, NSP and digestibility parameters. These findings were consistent with earlier work (Glencross et al., 2003). An assessment of the fibre composition of the kernel meals expanded on these findings and also showed that lignin was a key fibre class that affected protein digestibility, with higher lignin levels strongly correlating with poorer protein digestibility. That the digestibility of protein and energy can be shown to be related to certain compositional features of the lupin kernel meals allowed for the development of calibrations for near-infrared spectroscopy (NIRS) application (Chapter 8).

The digestibility of dry matter, crude protein and energy of the yellow lupin *L. luteus*, as well as of six of the new cultivars of *L. angustifolius* were determined when included in diets fed to the black tiger prawn (Chapter 25). In contrast to the results with the fin-fish there was comparatively less difference in the apparent energy digestibility (69.6% to 77.2%) and the apparent crude protein digestibility (92.7% and 96.8%).

Comparison of the digestibility of feeds and by inference, the ingredients, fed to either trout or Atlantic salmon showed that there was a high-degree of correlation in their responses to the different grain products (Chapters 19 and 20). However, correlation analysis between the various datasets also showed that there were some inconsistencies when comparing the results between different laboratories. The findings generally support that use of one species as an indicator of responses for another has some potential. But it is important to note that the data collection process has an important effect on the results achieved and to obtain the most viable cross-species data it is preferrable to have all experiments conducted by the same laboratory and personnel.

31.3.1.2 Anti-nutritional factors

Lupins do not have many anti-nutritional factors compared to most other legume feed grains (Petterson, 2000; Francis et al., 2001). Oligosaccharides and alkaloids could be regarded as the two anti-nutritional factors of most potential influence in lupins (Francis et al., 2001; Gatlin et al., 2007; Glencross et al., 2007). The work of this program examined the influence of a lupin alkaloid to both fish and prawns. The influence of the alkaloid gramine was shown to exert its anti-nutritonal effect through being a feed intake inhibitor (Chapter 9 and 27). The critical threshold for tolerance to gramine intake by rainbow trout was shown to be between 100 and 500 mg/kg of diet. Because prawns have a different sensory system to that of fish, the effect of the lupin alkaloid, gramine, when included in a feed for the black tiger prawn was also examined. The daily feed intake, growth rates and survival of the prawns were not affected by the concentration of gramine in the feed over the range of concentrations examined (0 to 902 mg/kg of feed, as used). However high levels of gramine did significantly reduce the feed intake of the prawns in the first 15 min after distribution of the feed. But, thereafter over the following 6 h that were closely monitored, feed intake did not appear to be affected by gramine inclusion level. It was noted that gramine leached from the prawn feeds quite rapidly with about 20% of the gramine lost in the first hour. These findings provides evidence that the alkaloid levels present in Australian domestic lupin varieties (< 200 mg/kg) are unlikely to result in anti-nutritional problems for fish. These data also show that there is significant scope for plant breeders to increase the gramine levels in the Yellow lupin from its current very low

level to levels that will provide much better protection against aphids, without compromising the nutritional value of the kernel meal.

A series of gut-health related issues were also observed with the inclusion of different grain protein raw materials in feeds for Atlantic salmon (Chapter 24). Ulcer-like lesions were observed in the stomach of fish from all feeding groups, and this was worsened by the presence of lupin in the diet. The distal intestine of fish fed soybean meal showed consistent and typical soybean meal-induced pathomorphological changes (Baeverfjord and Krogdahl, 1996), although no consistent altered morphology was observed in distal intestine of fish fed either fishmeal and lupin diets. It is believed that these pathomorphological changes induced by the inclusion of soybean in Atlantic salmon diets is a response to certain anti-nutritional factors (Krogdahl et al., 1995; Refstie et al., 2005).

31.3.1.3 Growth and utilisation

From growth studies it was demonstrated that fish can use lupin protein and energy as efficiently as that from fishmeal protein and energy, when diets are formulated and assessed on a digestible nutrient and energy basis.

The impact of variability in the digestible protein and energy content of lupin kernel meals was assessed in two separate growth experiments. The first experiment used low-protein diets (350 g/kg) and high-inclusion levels (40%) of a low digestibility and high digestibility lupin kernel meals and soybean meal. These diets were then fed at a restricted ration level and also to satiety to examine both palatability and utilisation aspects of the feeds (Glencross et al., 2007). The results demonstrated that a significant effect of the lower digestibility lupin kernel meal could be measured as an effect on growth. However, a second experiment examined the effect of the same raw materials at more typical inclusion levels (25%), in diets formulated to more typical commercial specifications (400 g/kg protein, 250 g/kg lipid). In this second experiment the effect of variability in digestible value was not as clear, demonstrating that under commercial equivalent conditions that variability in digestibility of lupin kernel meals would be unlikely to be observed, but that this built in margin-for-error adds significant cost to the diets.

The inclusion of lupin kernel and soybean meal in diets for sea-water reared Atlantic salmon was examined at two inclusion levels (15% and 25%) and at two water temperatures (14°C and 18°C) to examine if there was any influence of diet raw material on temperature response. An improved feed intake and growth response was observed from fish fed the lupin kernel meal diets compared to both the fish meal based reference and the soybean meal diets. This improved performance of the lupin kernel meal diets was observed at both water temperatures and is consistent with earlier grwoth studies with Atlantic salmon (Carter and Hauler, 2000). No interaction effect between temperature and diet/ingredient was observed in the study. These findings showed that lupin kernel meals have a significant advantage over soybean meal when included in diets for sea-water reared Atlantic salmon with the key response being improved growth from improved feed intake. It was also noted that the lupin diets also had maringally better growth than those fed the fish meal diet.

The growth performance of black tiger prawns when fed kernel meals of one of seven of the new cultivars of *Lupinus angustifolius* or solvent-extracted soybean meal was examined. In each experiment the growth rate of shrimp fed the diets containing lupin kernel meal or soybean meal was as good as, or better than that obtained with the fish meal based basal diet. These findings demonstrated that lupin kernel meal can be used to replace at least 40 % of the fishmeal protein in diets for *P. monodon*, and that the new cultivars perform equally to solvent-extracted

soybean meal when used on a protein-equivalent basis. From the amino acid analysis of the diets used in the experiments, it appears that that the reported requirements of juvenile prawns for the amino acid methionine significantly overestimate the true requirements. Further clarification of the actual amino acid requirements for prawns may allow a greater inclusion level to be adopted by commercial formulators.

31.3.2 Nutritional quality of protein concentrates and isolates

The program also developed and evaluated a range of protein concentrates and isolates when fed to rainbow trout and Atlantic salmon (Chapters 5, 12, 13, 14, 15, 16, 17, 19, 20, 21, 22 and 24). The dry matter, protein and energy digestibilities of the protein concentrates and isolates were high, but there was also subtle variation in the digestibility values depending on the actual methods used to produce them (Chapter 15). Protien isolates from any of the grains used resulted in products that had high protein and energy digestibilities. Protein concentrates produced from the same grains still had high protein digestibilities, but both these and their energy digestibilities were a little lower in comparison.

Growth of fish fed the protein concentrates was also good and demonstrated that these value-added grain products were well utilised and on a per unit protein-basis, as well utilised as fish meal protein (Chapter 16 and 17). However, significant inclusion of these products will need dietary supplmentation of amino acids to ensure no amino acid limitations are induced.

The influence of heat during protein concentrate drying was shown to not have a negative effect on the digestible value of lupin protein concentrates when fed to a fish, however, these heat-damaged protein concentrates were less palatable and did not sustain growth to an equivalent basis compared to spray or freeze-dried protein concentrates (Chapter 17).

31.4 Technical Development

The evaluation of raw materials is a central part of nutritional research and feed development for aquaculture species. It also forms the basis of identifying a prospective value for any raw material to this feed sector. Like most branches of science it is forever evolving and refining the technical aspects of the way in which it searches for new knowledge. In evaluating ingredients for use in aquaculture feeds there are several important knowledge components that need to be understood to support the use of a particular raw material in a feed formulation. This includes information on:

1 Ingredient digestibilities

2 Ingredient palatability

3 Nutrient utilisation and interference

The use of digestibility studies has played a central role in this research program (Chapters 4, 5, 7, 8, 9, 10, 11, 14, 15, 16, 17, 19, 20, 21, 22, 24, 25 and 28). Such an important role was given to these studies because this style of experiment allows for a high-throughput of samples and resulting data generation. The style of experiment also allows for the examination of prospective palatability issues during the diet acclimation phase that precedes the faecal collection part of the experiment. The use of different faecal collection methods remains a contentious one (Glencross et al., 2007). However, development work within this program has shown that faecal stripping is a more conservative option and that the differences between the determinations are exacerbated with the use of grain products (Chapter 14). It was also the preferred choice of

methodology by commercial partners, presumably because of its conservatism.

The application of near infrared spectroscopy (NIRS) to determine digestible nutrient and energy values is a significant technical advance in this scientific field. While the use of NIRS to measure digestible energy value from some grains fed to terrestrial livestock has been reported (van Barneveld et al., 1998), this is the first such assessment of a grain for an aquaculture species. Notably, in contrast to studies reported for terrestrial animals, the assessment undertaken in this program (Chapter 8) is the first to examine digestible nutrient and energy values from a single grain variety. Earlier work in pigs notably relying on the use of a range of different cereal types to allow the development of a calibration for digestible energy (van Barneveld et al., 1998).

The ability of fish to use nutrients from the test ingredient, or defining factors that interfere with that process, is perhaps the most complex and variable part of the ingredient evaluation process. It is crucial to discriminate effects on feed intake from effects on utilization of nutrients from ingredients (for growth and other metabolic processes). The work in this program has used a variety of strategies and explored some new ones to examine the effect that raw materials have on the interpretation of certain growth experiment designs (Chapters 9, 10, 11, 16, 17, 23, 26 and 27). To allow an increased focus on nutrient utilisation by the animals, several experiments examined the use of a bioenergetic method (Chapters 10, 11, 17). In other experiments histological methods were also used to examine any pathomorphologies associated with raw material use (Chapters 9 and 24). Other development aspects such as ingredient functionality were also important a consideration in determining the potential value of ingredients in aquaculture feed formulations.

The extrusion processing of feeds and the evaluation of the effects of different raw materials on the characteristics of the pellets is one such outcome (Chapter 29). Significant varibility in diet extrusion features was observed as a function of different lupin varieties/culitvars and also the actual species of feed grain being included in a diet. The inclusion of lupin kernel meals (from either *L. angustifolius* or *L. luteus*) was shown to increase bulk density, sink rate and pellet hardness and decrease vacuum oil uptake and pellet expansion, at a different degree than that achieved by a similar inclusion of soybean meal. However, the degree to which each factor was affected varied depending on grain product and its inclusion level.The identification of "functional" properties (also referred to as "technical" properties in some cases) provides an opportunity for increased value for a processed grain product (Glencross et al., 2007). Indeed, the functional value of lupins, along with their lack of key anti-nutrients and sound nutritional value are among the reasons why this grain is now being widely accepted in the aquaculture feed manufacturing sector. However, the extent of functional features that have been identified from grains in general has really only just been unsurfaced. It is likely that additional functional potential lies within lupins and other grains that is still to be identified and developed.

However, one the key issues with assessing functional properties that was identified from this program was the relevance of specific tests applied. While the use of some technical equipment may provide a means of collecting precise data, the implications of that data to actual functional features of grain need to be better established. This may require further work in exploring the implications of things such as the use of texture meter data and how this relates to milling issues with grain. However, similarly subjective is the assessment of fish pellet hardness and the implications of this on pellet durability, nutritional value and manufacturing constraints. Clearly this is a complex area that requires further thought and investigation.

Further technical development is still required to optimise the application of raw materials to use in aquaculture feeds. The high-level of competition among raw materials in this market demand

that each continue to define their "points-of-difference' and promote these and their benefits. The degree of application of science to this process is becoming more and more technical. It will be critical to maintain both scientific and marketing pressure to ensure certain raw materials maintain their industry adoption.

31.5 Adoption and Extension

The volume of knowledge now being generated on the application of grains to the aquaculture feed sector is considerable. However, there is an urgent need to collate this information to provide a comprehensive overview of the area, to highlight opportunities and identify knowledge gaps. Such a review was undertaken in 2001 by Glencross (2001), who reviewed all available publications on the application of lupins to aquaculture feeds. At the time this work proved to be an important promotional document that was intensively used by the grains industry to promote lupins, but also by the aquaculture feed sector to consolidate their confidence in the grain. Since this review (Glencross, 2001), significant advances have been made in the area of application to grains to aquaculture feeds. The work presented in this report exhibited several cases in point. The preparation of review documents provides not only a mechanism of promotion for the grains sector, but also a path of education for the users of grain and other researchers.

As part of the commercialisation process a series of studies were undertaken to examine the composition, digestibility and palatability to rainbow trout of different types of value-added grain products provided by two commercial collaborators. Details of each product and their assessment were conducted on a commercial-in-confidence basis and as such no details will be provided. A total of eight products from both companies (CBH-Group and Weston Technologies) were evaluated over a two-year period. This practice, while useful in obtaining data for the commercial operators to start promoting their own products has little scientific value.

An additional part of the commercialisation process involved the collation of samples and data from large (n=75) sample set of lupin meals. In this component, samples were collected, prepared, analysed and evaluated for their digestible energy and nutrient values and a sample provided to each of the participating commercial partners (Chapter 30). This data was then supplied to each of the collaborating commercial partners, along with samples of the kernel meal, to allow the development of calibrations for chemical and nutritional properties using near infrared spectroscopy (NIRS). Notably, each collaborator had different NIRS equipment and accordingly required their own sample set to allow the development of calibrations. Perhaps one of the greatest strengths in this sample/data set is the long-term retention of samples for future data mining or further assessment.

As part of the program, Skretting Australia, the largest aquaculture feed manufacturer in Australia has broadly adopted the use of lupin kernel meals across their product range. The adoption of the raw material has also spread further within this multinational group, with companies within the Skretting group in Norway, Japan and Chile also adopting the use of lupin kernel meals. The close integration of a commercial partner such as Skretting has been critical to the success of this program in commercial extension of value-added feed grain use in aquaculture feeds. The strategy of working closely with a single company has also resulted in other feed companies in Australia, and internationally following the lead of Skretting and also commencing adoption of the use of lupin kernel meals. In getting such as "slip-stream" effect to work it has been important to have a market-leader as a commercial partner in the program.

In the present program a series of niche promotion visits were made to key domestic and

international trade-markets (Norway, Chile, China, Canada, Japan, Thailand, Vietnam) on both a strategic and opportunistic basis. This approach had many advantages in that it assisted the development of confidence in the technology behind the raw material development through the development of relationships between the user, researcher and grain processor. It also allows for the identification of any key concerns the market had so as the research could be better targeted to addressing those specific issues in the intent of overcoming any trade hurdles.

The other key component to the value-chain in this value-adding process has been the invovlement of commercial grain processors. Drawing from the work in this project, both CBH-Group and Weston Technologies have formed a joint-venture company to develop a 200,000 tonne per annum lupin kernel meal production facility. The joint-venture company, Australian Lupin Processing Pty Ltd commenced production in early 2007. The targetting of lupin kernel meals to the aquaculture market was highlighted as one of its key initiatives. By engaging these two major grain industry companies significant resources and momentum were able to be directed at this initiative.

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32.0 Recommendations – The Future of Feed Grains Development for Aquaculture

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Abstract

A series of projects were undertaken to both develop the potential of lupins as a feed grain for the aquaculture sector, and to also facilitate the adoption of this grain by this feed sector. The outcome of these activities has been the creation of a new, higher-value market for lupins based on a local, value-adding industry and to also reduce resource risk for the aquaculture industry by reducing their reliance on fish meal as a protein source. However, because of the broad-base approach of this project there have been numerous issues identified that need further addressing on one format or other. These issues can loosely be defined as being pertinent to grain sector issues or aquaculture sector issues.

The grain-sector issues include the need to introduce a grain segregation system to maximize the value of higher-protein crops and also improve returns to producers. This process can be better encouraged by the release of high-yielding, high-protein lupin varieties, including development of better agronomic packages for *L. luteus* and *L. mutabilis*. There are also opportunities for grain varieties other than lupins in the aquaculture feeds sector, though all grains require some degree of value-adding to be competitive raw materials in this market. Pursuit of new, novel processing technologies and refinement of processing techniques to isolate specific functional and nutraceutical products from grains are an area that warrants some attention. Establishment of new markets for grain products will require considerable extension but represents a worthwhile investment area to improve market penetration of grains in to aquaculture feeds.

For the aquaculture feed sector there is a need to improve our understanding of the functional chemistry of the nutritional variability of grains on fish. With the increasing use of grains, the inadvertent inclusion of carbohydrates in fish diets will introduce a range of effects. There is a need to examine these effects to define what roles, if any, that certain carbohydrates play in fish digestion and nutrition to tailor optimal use of grain resources. This will have roles in examining the digestion, gut microflora, nutrient demands and functional properties of fish and fish feeds. The inclusion of products with nutritional and health benefits (nutraceuticals) derived from grains and other plant products is an area of significant potential and further work in this area is warranted. However, despite the technical capacity, and in some cases also economic capacity, to replace almost all the fish meal in aquaculture diets, the commercial advent of "fish positive" feeds, where the fish content of a feed is reduced to below 25% and therefore produces more fish than it consumes is still to be realised. The key limitation to this appears to be industry acceptance, with popular belief that fish feeds have to contain fish protein or fish oil still persisting. Therefore significant extension work is required to educate the users of fish feeds and the broader community on the realities and possibilities of grain use in fish feeds.

32.1 Introduction

The research presented in this report was done with the intent of improving our understanding of the nutritional characteristics of a range of grain resources, but with a specific focus on lupins and their potential for aquaculture feeds. However, like all research programs, the end-point is rarely black-and-white. Progress achieved in certain areas, highlights deficiencies in others, new findings in one area point to new leads in another.

Many of the outcomes achieved from this research have already strengthened the position of grain products in general and lupins in particular, as ingredients to be used by the aquaculture feeds industry. The outcomes have also served the grain processor's interests by clearly defining some of the quality criteria that will be important to the aquaculture sector. In the process of answering many of the questions posed in addressing the two key challenges, many other issues have also arisen that require further attention to better facilitate the adoption of this technology and thereby improve the potential market penetration for feed grains and reduce the risk associated with fish meal use in aquaculture diets.

Because of the broad-base approach of this project there have been numerous issues identified that require further addressing on one format or other. These issues can loosely be defined as being pertinent to the grain sector or to the aquaculture sector.

32.2 Further Grain Development

There is a range of recommendations to be made about the further development of grains of use in the aquaculture feed sector in particular and the broader animal feed sector in general. These recommendations have been categorised according to: grain production development, grain quality management, grain processing and grain promotion.

32.2.1 Grain production development

The use of kernel meals of *L. angustifolius* proved to be one of the most viable value-added grain products evaluated in this program (Glencross, 2007). However, kernel meals from this lupin species are only just viable (both economically and technically) and grain-product protein levels lower than 38% risk its non-inclusion in many aquaculture formulations through an inability to fit within key formulation constraints (Glencross, 2003). Therefore any progress towards increasing protein content of *L. angustifolius* varieties without a loss in crop yield would be a significant advance. Furthermore, despite some preliminary findings on the genetic and environmental influences on *L. angustifolius* quality (Cowling and Tarr, 2004; French, 2005), further progress in this area to improve the potential of the producer in manipulating grain quality will also be a significant advance.

The identification that an ideal grain protein product has between 50% and 60% protein clearly prioritises the potential for kernel meals from *L. luteus* and *L. mutabilis* lupin species (Glencross, 2003; Glencross et al., 2004c). Production of kernel meals remains the most economically viable form of value-adding for lupins and therefore products from these two lupin species are the only products that can fit within this product specification. However, the limiting factor in development of a viable grain value-adding sector based on these lupin species is the production capacity for either grain within the Australian farming system. Presently neither *L. luteus* nor *L. mutabilis* are produced in significant quantities to justify an end-user committing to their use and without an end-user like the aquaculture feed sector committing to a significant tonnage

at a viable price per tonne of grain then farmers are reluctant to produce the crop, hence a case of market failure is occurring. To overcome this there is an urgent need for not only the promotion of higher-protein varieties of *L. angustifolius*, but also the development of improved "farm-production-packages" for existing varieties of *L. luteus* such as Wodjil or Pootalong. Increased production of *L. angustifolius* cv Coromup would be a favourable outcome, even though this variety is still relatively uncompetitive as a feed product against either *L. luteus* or *L. mutabilis*.

The higher-protein lupin species also provide significant advantages in the production of protein isolates and concentrates in that they provide a higher baseline from which to start from and therefore either increase the protein content in the end product and/or increase the yield. For this reason, further advances in protein product development will be well placed to focus on these grain varieties as their base material and rely on *L. angustifolius* as a material of second-choice.

One avenue of improving the farming viability of *L. luteus* is to improve its resistance to insect infestation (Berlandier and Sweetingham, 2003; Risdall-Smith et al., 2004). This could be advanced through the reintroduction of certain alkaloids to the plant, but being mindful of thresholds applicable for the subsequent use of the grain in animal feeds. Recent work has demonstrated that fish are among the most sensitive of production animals to the influence of alkaloids on feed intake, with a threshold between 100 and 500 mg/kg of the diet (Glencross et al., 2006). By increasing the alkaloid content of the plant such that the grain has a limit of 500 mg/kg may aid in improving the plants defence against insect infestation, and will easily allow a 20% inclusion of the grain without introduction of anti-nutritional effects on the animal to which the grain is being fed.

Another option for improving the grain production yield reliability of *L. luteus* and other lupin species may be through targeted genetic improvement. The use of transgenic technologies has some potential, though the market risk and advantages of using or not using the technology must be weighed up. With lupins transgenics has been used to modify the amino acids structure of the seed, although this has been shown to not produce any significant commercial benefits, despite that a measurable effect of the extra methionine (Glencross et al., 2003). The use of this technology would be better directed towards issues such as improve yield characteristics, higher protein levels or the inclusion of functional or nutraceutical properties in the seed.

Primarily through the insistence of the commercial partners in the program, the work in this report has focussed heavily on the application of lupins as a value-added grain. However, there remains considerable scope for the development of other grains such as peas, beans, cereals and canola to also provide some prospect for value-adding and the development of products suitable for use in the aquaculture feed sector (Gatlin et al., 2007; Glencross et al. 2007a). This may also provide opportunities for increasing the value of any such grains entering this feed sector above those paid in other animal feed sectors.

32.2.2 Grain quality management

That lupin kernel products are now being actively utilised by the domestic and international aquaculture feed sector, it is important that grain quality assurance is maintained to ensure market security. The key aspect to maintenance of this market (aquaculture feed sector) is ensuring that sufficient product is available, even in times of drought and that its protein levels are kept as high as possible, certainly above 38% protein.

The ensuring of sufficient product will be somewhat affected by uncontrollable climatic factors, though even in drought years there is always some grain available and obtaining this to process to ensure that key customers are serviced should be a priority. The risk in losing a customer through lack of product is that they will replace the product with an alternative and in future have to be encouraged to take lupin kernel meals on again.

Maintaining quality standards could be achieved by segregation of higher-protein grain (Kingwell, 2005). The grain could be assessed on receival and farmers paid to store the grain on-farm or it allocated to excess storage at receival points. This more valuable grain would clearly attract a premium. The extent of that premium varies according to many independent market factors. The near-infrared spectroscopy (NIRS) calibration developed in the present program also has a clear place in this option as the original seed stocks were also retained. This will allow the development of NIRS calibrations for kernel meals based on the assessment of seed. Ultimately this will allow assessment of kernel meal characteristics at receival points to assist with the segregation process.

Throughout this work there have also been discrepancies identified in the assessment of lupin kernel meal protein. Based on the standard method of protein assessment (nitrogen x 6.25) and the assessment of the cumulative amount of amino acids (sum of amino acids) the later always compares to be less than that value arrived at based on the nitrogen value. This suggests that either the correction factor is inappropriate (and a new one of 6.02 has been suggested). However, based on the variability seen among varieties based on the two protein assessment methods there has also been the identification of a certain component of non-protein-nitrogen (NPN). Presently it is not known what this specifically may be, but at the suggested levels can be appreciable (~0.5% of the DM as NPN). Further assessment to define what this NPN would be useful and warrants investigation.

The use of NIRS to assess grain quality is a highly useful tool. The development of NIRS calibration for the assessment of digestible protein and energy from a grain is a significant advancement for both the grains processing and the aquaculture feed sector (Bertrand, 2001). The present study based on the assessment on 75 samples resulted in some of the calibrations being marginally non-significant and would probably become viable calibrations with additional 25 or so samples. A valuable aspect of this NIRS calibration is not only the assessment of the nutritional values of a range of lupin kernel meals, but as much the retention of samples for future analysis as a repository of kernel meal variability with corresponding chemical analysis. This sample set, which has been made available to the commercial partners in the program, forms the basis of a comprehensive evaluation of many of the quality criteria of this product. In this regard, using this sample set for further chemical analysis to more fully evaluate the sample set would be worthwhile.

32.2.3 Grain processing

There will also be some post-processing opportunities to improve the overall value of lupin kernel meals. By segregation of higher protein lupin species and varieties the capacity to blend different kernel meals to produce meals that always conform to certain specifications will be achievable. This blending approach could also be used to ensure that a grain processor always maintained a competitive edge over any competition, by being able to ensure a more reliable composition and also prospectively "outbidding" the competition on protein content of their product.

Any options to improve the efficiency of lupin dehulling would be well worth exploring. Not only will any gains in dehulling efficiency improve the nutritional value of the value-added grain product produced (Glencross et al., 2007b), but also increase the overall protein content

and therefore its direct marketable value. However, the downside is that increased dehulling efficiency will be likely to decrease the yield of kernel product and therefore any gains in quality have to be offset against changes in yield. There is likely to be a point-of-marginal returns that could be calculated depending on the price being paid per unit protein, the costs associated with increased dehulling efficiency and changes in yield.

The identification of "functional" properties (also referred to as "technical" properties in some cases) provides a mechanism for increased value for a processed grain product (Sipsas, 2005). Indeed, the functional value of lupins, along with their lack of key anti-nutrients and sound nutritional value are one of the reasons why this grain is now being widely accepted in the aquaculture feed manufacturing sector. However, the extent of functional features that have been identified from grains in general has really only just been unsurfaced. It is likely that additional functional potential lies within lupins and other grains that are still to be identified. To assess this further specific tests will need to be applied in the assessment of "point-of-difference" features from physical and chemical properties of the various grains. In an aquaculture feeds perspective this will clearly require further assessment of extrusion processing technology and exploration of the effects of various grains, their inclusion levels and the effects on pellet structure.

However, one of the key issues with assessing functional properties that was identified from this program was the relevance of specific tests applied. While the use of some technical equipment may provide a means of collecting precise data, the implications of that data to actual functional features of grain need to be better established. This may require further work in exploring the implications of things such as the use of texture meter data and how this relates to milling issues with grain. However, similarly subjective is the assessment of fish pellet hardness and the implications of this on pellet durability, nutritional value and manufacturing constraints. Clearly this is a complex area that requires further thought and investigation.

One problem identified with lupins was their poor ability to be moved as a bulk commodity. Lupin kernel meals tend to be highly hydroscopic, do not flow well and become sticky with the application of heat. Therefore the development of simple methods to compound the meals into "lupin nuts" through the use of steam- or compression pelleting of the kernel meal may provide an option for logistics management to improve handling of lupin kernel meals. The development of "lupin nuts" may also not only be used as a means of improving bulk trade options (bulk density, flowability, stickiness), but also provide opportunities for the addition of anti-oxidants and anti-fungals to reduce the threat associated from rancidity and mould susceptibility. However, pilot-scale trials are need to validate the economic viability of such a process.

Further assessment of the variability in grain hardness among lupin species and varieties also needs to be explored to determine whether there are species or varieties that have milling properties that make them more suitable to value-adding. However, the technology to examine these needs to be revisited as use of texture meter assessment, as was reported in earlier work (Chapter 6), lacks any confirmed linkage to the effects likely to be seen during commercial milling of the products. Development of a small milling system that has the capacity to measure energy demand or throughput should be sufficient to examine this issue. Though even this will require benchmarking against commercial mills. This work could be extended to examine possible engineering solutions to improve the milling efficiency of lupins, though an initial examination of different existing milling strategies would be a useful starting point.

The techniques used in this report to develop protein concentrates and isolates are based on already published and widely used methods (Lasztity et al., 2001; Sipsas 2003). However, there

are likely to be other processing technologies that may be applicable and new opportunities are also likely to arise over time as the advent of other new equipment arises and new economic opportunities occur. Some investment in further exploratory processing technologies may be warranted. As is the application of co- and by-products to further assist the economies of protein concentrate production.

The key avenues to improving the viability of protein concentrate and isolate development will be to increase the protein yield (value) of the product and/or reduce the associated drying costs. These two factors have already been identified as important viability limiting steps (Kingwell, 2003). Further development of new extraction techniques may improve the yield, but drying costs are likely to be strongly linked to the cost of energy. The development of new technologies for lowering drying costs or perhaps increased throughput through existing drying processes is two options to be aware of.

One aspect of the further exploratory technologies would be in the specific isolation of functional materials from grains. This could be in the form of products that improve the physical characteristics of a fish pellet, such as their binding strength. However, lupins, like most plants have a variety of biologically active compounds as part of their make up, like isoflavonoids (Petterson, 2000), and the identification and concentration of compounds that enhance the nutritional and/or health aspects of a fish feed could be a further means of increasing the value of the grain. A mechanism of screening a range of products for functional and nutraceutical activity is needed to enable the assessment of a wide variety of samples. Such a screening mechanism may be in vitro, but should be referenced back to an in vivo assessment to make sure that it maintains relevance. Development of in vitro assays for assessment of raw materials previously has not been overly successful and requires more work (Carter et al., 1999; Rungruangsak-Torrissen et al., 2002).

Other grains also provide opportunities for value-adding. The value-adding of field peas in particular has some prospect where a co-product stream of a pea protein concentrate and a pea starch can be produced. Similar such options may also be available from Broad/ Faba beans (Gatlin et al, 2007). The production of starch and gluten enriched products from a range of cereals is already a widespread industrial process. However, like many of these intensive value-adding technologies they are costly and the viability of widely using the product in animal feeds is limited. Canola also may lend itself to further protein concentration or isolation because of the technologies used to extract the target product of oil. As a co-process canola meal could be directed through additional extractive processes to remove the fibre and value-add the meal. Preliminary studies have already examined some of these opportunities, but have indicated that further development is required (Glencross et al., 2004a; 2004b).

The development of a biofuels industry also provides some opportunities for further development of grain value-adding. The use of cereal grains for ethanol production produces a by-product referred to as dry distillers grains solubles (DDGS). Some work has already been undertaken internationally examining the potential of using this by-product in its existing form (Tidwell et al., 2000), though the product also lends itself to further post-processing to concentrate the protein content further. Canola also fall into this category, especially seeing as they are showing some potential as a feed stock for bio-diesel production. The by-product in this case would still be a canola meal, but the off-set created by a higher-priced primary product (the oil) further improves the scope for lowering the cost of the canola meal and making the value-adding of it more attractive.

32.2.4 Grain promotion

There is extreme competition in the international market place for raw materials. Because of this competition, actively lobbying and marketing is important in gaining acceptance of raw materials in most markets. There are several ways this can be achieved, including passive promotion through the publication of scientific and industry articles in various forums, through to active promotion by site visits and face-to-face meetings with key individuals in key markets.

In the present program the niche promotion to key trade-markets (Norway, Chile, China, Canada, Japan, Thailand, Vietnam) was undertaken both strategically and opportunistically. This approach has many advantages in that it engenders confidence in the technology behind the raw material development through the development of relationships between the user, researcher and grain processor. It also allows for the identification of any key concerns the market may have so as the research can be better targeted to addressing those specific issues in the intent of overcoming any trade hurdles. However, in some situations the key trade hurdles will be trade tariffs, in which case diplomatic and political pressure needs to be engaged to improve terms-of-trade.

Another option that could be used to promote the use of grains to target markets is the preparation of summary sheets and reviews in the target market languages. There is a range of such reviews available and summary sheets have been prepared previously that may be amenable for translation. The use of the internet for dispersing this information could also be promoted, though getting the market aware of the resource usually requires some additional approach such as advertisements in industry publications or leaflets at trade shows.

Presently the American Soybean Association (ASA) has a very proactive extension program operating in Asia (American Soybean Association, 2007). Through on-farm demonstration trials the ASA is using a research presence to increase the exposure of the rapidly growing aquaculture industry in this region to soybean meal use a quality feed raw material. Through this initiative there is the opportunity for the Australian grain sector to 'piggyback' on this work demonstrating that not only can soybean meal be used, but that other grains, like lupins are also viable aquaculture feed options.

32.3 Further Aquaculture Development

The aquaculture sector has a different spectrum of needs arising from issues identified in this program. The use of significant quantities of grains in aquaculture feeds is only now beginning to establish itself in Australia and internationally (Gatlin et al., 2007; Glencross et al., 2007a). Many prospective issues with the application of grains to fish feeds are probably yet to surface.

32.3.1 Nutritional Development

One of the consequences that will occur with this increased use of grain is a significant increase in the carbohydrate content of fish diets, ironically an animal class that is generally poorly adapted to the metabolism of these molecules (Hemre et al., 2002). The higher levels of carbohydrates (CHO) are likely to introduce responses from changes in gut bacterial proliferation, digesta viscosity, changes to glycaemic control and energy balance and also the introduction of xeno-compounds. It may even be possible that some of these responses may be beneficial.

There is a need to further characterise the functional chemistry of nutritional variability associated with the inclusion of different types of CHO in diets for fish. This could be

undertaken in a range of ways, but a cross-referencing approach of wide use of different grain resources and subsequent use of multivariate statistics to identify key influential CHO would be a useful starting point. This could then be cross-referenced with a directed approach with the inclusion of purified CHO in diets to see if a predicted response can be achieved. A range of response variables could be included in such work, ranging from physical parameters of feeds, to metabolic and histological responses of fish to more fundamental digestibility and growth responses. However, this work would still have to consider the compositional complexities even within CHO classes.

An additional parameter that has had scant work committed to it in this field is the influence of raw material choice on generalised gut function and also the proliferation of gut microflora. Recent work has shown that there is considerable complexity in gut microflora in fish and that this may be influenced by feed type and the raw materials used in certain feeds (Ringo et al., 1999).

It has been frequently pointed out that the use of grain resources in aquaculture feeds regularly introduces a suite of anti-nutritional factors (Francis et al., 2001; Gatlin et al., 2007; Glencross et al., 2007a). These anti-nutritional factors (ANF) are in essence biologically active compounds that were evolved by plants to limit themselves from being eaten by animals. While the extent of published work on the influence of these ANF on fish is increasing, further work is still required in this area. In addition to the ANF aspect of these biologically active compounds, there is also the prospective nutraceutical potential of some of these ANF and other biologically active plant compounds that may have certain commercial potential for improving fish production efficiencies.

The observation that there was considerable variability in digestible value of protein and that this was affected by both the protein content and lignin content of the grain also supports further examination of the issue of protein quality. This could be examined by further studies on the influence of protein class variability and the digestible / nutritional value of grain proteins when fed to fish. Clearly some capacity remains to utilise the lupin kernel meal reference set to explore the variability of protein classes and see if this relates to differences in nitrogen or sum of amino acids digestibilities.

The introduction of alternative protein sources, such as feed grains, can ultimately introduce amino acid limitations into the diets of species to which they are being fed. Because many feed grains are relatively deficient in either or both lysine and methionine, these two amino acids are key nutrients of concern with increasing application of grains in feeds. Although the inclusion level of grains in a feed would have to be substantial to induce such a limitation, improved knowledge of key amino acid requirements will engender confidence among formulators to ensure that possible amino acids limitations are not encroached. For some species, limits in the formulation of diets to the inclusion of certain feed grains are included because of perceived limitations that the use of these grains introduces with respect to amino acid requirements (Fox et al., 2007). Indeed, based on existing premises that a fish's amino acid requirements reflect the proportions of all essential amino acids relative to the first limiting amino acid and the metabolisable energy content of the feed, then it is likely that limitations to several amino acids maybe encroached on, and as increasing levels of fishmeal substitution are to occur the risk of such limitations occurring are likely to increase.

It is also apparent from the literature and the work in this program that there can be markedly different outcomes for the same grain product, but with application in different markets (e.g. prawn feeds vs salmon feeds). In this regard it is important to consider that further grains work needs to be mindful of its target market for the grain being assessed. While the initial

development work presented in this report has some broad implications for fish in general, specific access to certain markets is likely to be limited by the availability of data on certain grains when fed to the species in those markets. Key examples of this include the use of grains in feeds for catfish in Vietnam, shrimp in Thailand or marine fish in China.

32.3.2 Technical Development

The development of a near-infrared spectroscopy (NIRS) calibration for the assessment of digestible protein and energy from a grain is a significant advancement for the aquaculture feed sector. The calibration developed within this program is the most intensive such study ever conducted for the aquaculture feed sector on a feed grain. However, the lack of a high-level of variability in grain composition means that a greater number of samples are required to increase the robustness of the calibration. The present study committed to the assessment of 60 samples and delivered an assessment on 75 samples. Many of the calibrations are marginally non-significant and would probably become viable calibrations with additional 25 or so samples. Perhaps one of the most valuable aspects of this NIRS calibration development is the assessment of the nutritional values of a range of lupin kernel meals and the retention of samples for future analysis. Presently reference samples of those lupin kernel meals already evaluated are being maintained in cold storage at the Department of Fisheries Marine Laboratories in Hillarys, WA. It is intended that these samples will be maintained for future analytical and reference requirements.

All raw materials exert some influence on the functional properties in any feed pellet in which they are included. These influences can be either positive or negative. Those influences that are positive can create not only market advantage, but are in effect also worth an increase in the relative value of the raw material on a $/unit protein basis.

The work presented in this report shows a small examination of the influence of different grain value-added products on the functional aspects of feed extrusion, the primary means of production of fish feeds. However, only a limited amount of resources was directed at this initiative and further work on this area may be warranted. The present study simply examines the effects of serial inclusion of the different grain-products into a fish feed, but without balancing of the diets for various typical formulation constraints such as starch and protein levels. While the present study does allow for the discrete examination of the effects of each grain-product on the extrusion process it needs to be further evaluated with what would be a more practical approach in maintaining starch and protein levels and certain thresholds. By further examining the flexibility of some of the functionality features under formulation variations, it may be possible to further identify other means of manipulating extruded product features to create other product advantages.

Although most fish feeds are made using extrusion technology, shrimp feeds are still produced using steam-pelleting technologies. There is a need to examine the effects of grain-product inclusion on the functionality of pellets produced using this technology (Smith, 2007). This knowledge may be pivotal to gaining acceptance of the product in shrimp feeds in Southeast Asia.

32.3.3 Aquaculture Extension

Despite the technical capacity, and in some cases also an economic capacity, to replace almost all the fish meal in aquaculture diets, the commercial advent of "fish positive" feeds, where the fish content of a feed is reduced to below 25% and therefore produces more fish than it

consumes is still to be realised (Naylor et al., 2001). The key limitation to this adoption appears to be industry acceptance, with popular belief being that fish feeds have to contain a certain amount of fish protein or fish oil for fish to grow well. To address this problem there is a need for a series of industry-hosted trials assessing the performance of feeds formulated and manufactured with high-levels of fishmeal replacement. Clearly prior laboratory assessment of a series of test options would be prudent before under-taking such industry based assessments. In addition to the conduct of a series of on-farm tests the broader education of the production sector on nutritional management and what can and can't be achieved in feed manufacture is also needed. However, this education process may be better serviced by the feed production sector that could use it as mechanism of customer service and loyalty development.

The specific extension of fishmeal replacement technology to fish farmers (feed users) is not perceived to be of high priority. Most farmers are oblivious to the content of their feeds and would be better served by educating them to the nutritional implications of variations in feed composition than formulation options. In most cases there is significant variation in raw material options in formulations across feed mills and even within feed mills but across time, so as to make the education of farmers about the risks associated with such raw materials of little value. Any education or extension effort on raw materials would be better served by maintaining the focus at the feed mill and formulator.

The development of the NIRS calibration also provides opportunities for product extension. By provision of samples and data associated with those samples grain processors could provide advantage to select customers choosing to optimise their use of lupin kernel meals. This may open opportunities for access to new markets. Clearly the key value in this example is in the retention and maintenance of the reference sample set.

Access to certain markets is likely to be limited by the availability of data on certain grains when fed to the species in those markets. As formulators for those species are likely to seek assurance of the grain's potential when fed to the fish they are formulating for. Key examples of this include the use of grains in feeds for catfish in Vietnam, shrimp in Thailand or marine fish in Japan. In this regard it is important to consider that further work to increase export of grains for this market sector may require the need for a series of extension trials in the target market country to demonstrate the potential of the grain being marketed. Such work may be best suited to closed market arrangements between a grain exporter and specific target market companies.

32.4 Cross Sector Development

Although many of the recommendations already made are relatively specific to either the grains sector or the aquaculture sector, there are also issues that straddle the needs of both sectors. The promotion to and education of both grain processors and feed manufacturers will improve the potential for value-added grains to penetrate this market. By improving the knowledge of grain processors on key issues such as the comparative value of different grains, formulation constraints of different grains and diets and processing issues associated with using grains in extrusion systems, their ability to market grains to this sector will be significantly improved. Conversely the feed manufacturing sector could also gain from improving their knowledge on key issues such as the comparative value of the different grains, the implications of processing on feed grain quality issues and also the implications of different grains on extrusion processing issue. This knowledge would improve the feed manufacturers confidence in grain products and increase their ability to confidently use grains as a raw material.

32.4.1 Cross Sector Extension

The expansion of markets for lupin kernel meals or any other grain product for use in the aquaculture feed sector will require significant trade development. The American Soybean Association presently heavily invests in the promotion and extension of soybean use in this sector (ASA, 2007). While these efforts will also pave the way for the entrance of lupin kernel meals, a process of confidence building in lupin kernel meals and soybean displacement would be required.

To engage with target markets the most practical way to extend the knowledge gained is through hosted trial work. In this scenario the grain processor / trader collaborates with the grain user to sponsor a trial, of which the outcome is largely already known by the grain processor, based on prior work. The outcome of the collaborative trial then forming the foundation for further trade. Once the grain user / feed manufacturer has garnered confidence in the product and adoption has occurred there is likely to be "cross-fertilisation" of the use of lupin kernel meals across feed mills based on staff movement and market intelligence. Clearly to initiate this process there is a need to the coordinated conduct of evaluation trials in select target markets.

The volume of knowledge being generated now on the application of grains to the aquaculture feed sector is considerable. However, there is an urgent need to collate this information to provide a comprehensive overview of the area, to highlight opportunities and identify knowledge gaps. Such a review was undertaken in 2001 by Glencross (2001), who reviewed all available publications on the application of lupins to aquaculture feeds. At the time this work proved to be a major promotional document that was used by the grains industry to promote lupins, but also by the aquaculture feed sector to consolidate their confidence in the grain. Since this review (Glencross, 2001), significant advances have been made in the area of application to grains to aquaculture feeds. The work presented in this report being cases in point.

The preparation of review documents provides not only a mechanism of promotion for the grains sector, but also a path of education for the users of grain and other researchers.

32.5 Conclusion

Through this program there has been an intensive effort to collaborate with both the grain processing and aquaculture feed sectors to engender their mutual confidence in the use of lupin kernel meals as raw material for the aquaculture feed market. Like all research and development processes there is no discernable end-point as the resolution of old problems unearths more new ones.

The key to the success of a program such as this one has been through the broad extent of its collaborative engagement and this has allowed it to establish the required linkages for the adoption of these new products and process to occur. In this regard the program is a complex engagement that has had to deal with the requirements and interests of many stakeholders and attempt to achieve this in the context of a research program. It may serve as a viable model for future such initiatives.

32.6 References

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