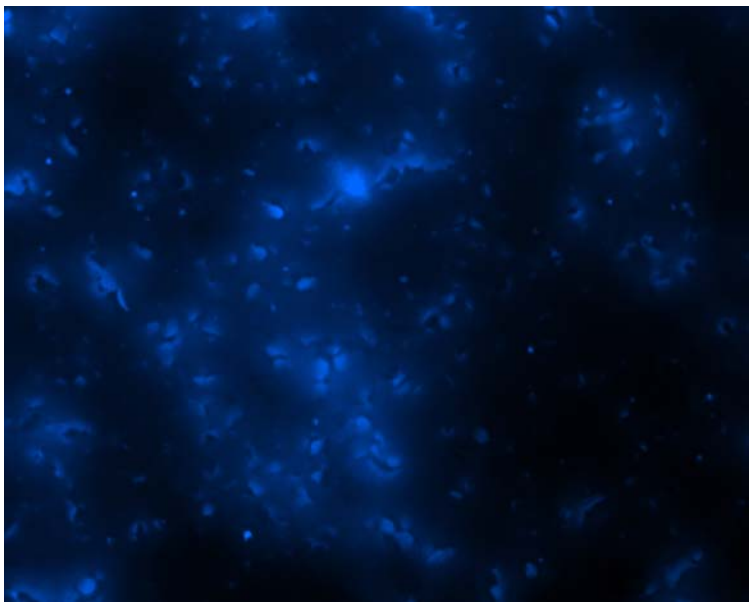
Desmozoon lepeophtherii as a pathogen in Norwegian salmon aquaculture



Thesis for the degree of Master of Science in Aquamedicine

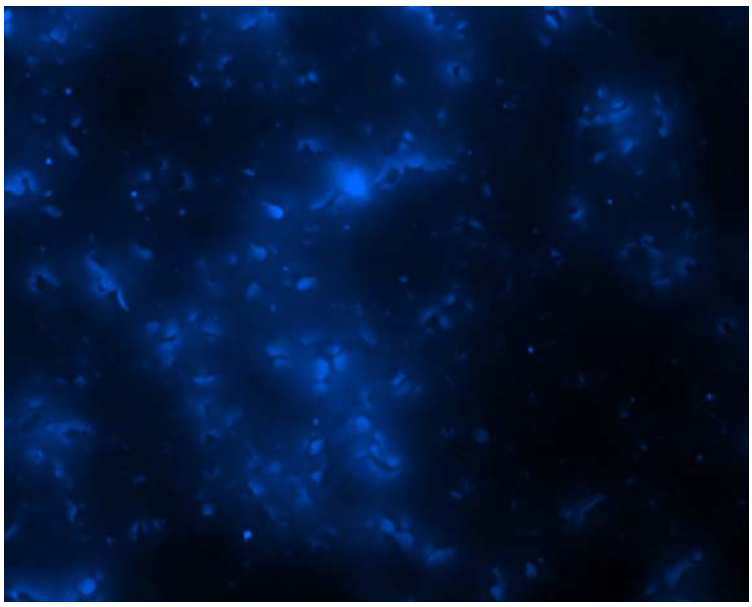
Miriam Nerland Hamadi

Department of Biology

University of Bergen

June 2011

Desmozoon lepeophtherii as a pathogen in Norwegian salmon aquaculture



Thesis for the degree of Master of Science in Aquamedicine

Miriam Nerland Hamadi

Department of Biology

University of Bergen

June 2011

Abbreviations

ASPV	Atlantic salmon paramyxovirus
CT	cycle threshold
CMS	cardiomyopathy syndrome
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphopate
EF1A	elongation factor 1a
FFPE	formalin fixed paraffin embedded
HSMI	heart and skeletal muscle inflammation
KOH	potassium hydroxide
NAC	nucleic acid concentration
NSAV	Norwegian salmonid alphavirus
NTC	non-template control
NUC	targeting gene *D. lepeophtherii*
NVIB	Norwegian Veterinary Institute Bergen
NVIH	Norwegian Veterinary Institute Harstad
PCR	polymerase chain reaction
PD	pancreas disease
PGI	proliferative gill inflammation
PMCV	piscine myocarditis virus
PRV	piscine reovirus
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
ROX	passive reference
RT-PCR	real time polymerase chain reaction
SAV	salmonid alphavirus
SGPV	salmon gill poxvirus
TEM	transmission electron microscopy
UoB	University of Bergen

Acknowledgements

This thesis is written as a part of the degree of Master in Aquamedicine carried out at the University of Bergen at the faculty of mathematic and natural sciences. The study was performed at the Norwegian Veterinary Institute Bergen (NVIB), Norway.

First of all I would like to thank the Norwegian Veterinary Institute Harstad and Bergen for making this project possible, and to let me take part in such an interesting project. There are a handful of people I would like to show my gratitude to. Special thanks to my teaching supervisor Øyvind Brune Vågnes for giving me a fantastic guidance in the writing process, and giving me wise advices through the whole year at NVIB. Cecilie Sviland and Geir Bornø from the Norvegian Veterinary Institute Harstad and Niels Petter Maaseide from Intervet Norway for the collaboration on my project. Thanks to Frank Nilsen for his willingness to be my second supervisor at UoB.

I also want to express my sincere gratitude to the bioengineering staff at NVIB, for teaching and helping me with the use of the laboratory equipment, answering my lab-questions and helping me preparing the tissue sections for calcofluor. Thanks to Anne Berit Olsen for reading this assignment for a final correction. I must also thank Hege Avsnes Dale at the Molecular Imaging Centre, at UoB, for helping me with the microscope technical part and capturing pictures of the calcofluor material.

After a six year period of studying I have made a lot of good friends. In difficult periods we have always stood up and supported each other. The memories and good times as students will always be remembered. Thanks to my fellow masterstudent here at NVIB, Marte Kalve Reigstad, who always was there for me with a comforting shoulder, and for calming me down when needed.

Last but not least, I would like to thank my family for helping me through the long and stressful period from the first step on my plane to Bergen in 2005, to the final day of graduation.

Bergen, June 2011 Miriam Nerland Hamadi

Contents

Abstract

During late summer and autumn 2008 the Norwegian Veterinary Institute Bergen received several diagnostic cases with similar histopathological changes. Due to unknown cause of the the condition it was subsequently called “autumn disease”. Later on the microsporidian *Desmozoon lepeophtherii* was proposed associated with the autumn disease. *D. lepeophtherii* was also suggested associated with the development of pancreas disease (PD), heart and skeletal muscle inflammation (HSMI), cardiomyopathy syndrome (CMS) and proliferative gill inflammation (PGI) in farmed Atlantic salmon.

In this study real time PCR and calcofluor white stain were used to detect *D. lepeophtherii* in formalin fixed paraffin embedded organ tissue used for diagnostic purposes. This was material and fish samples officially diagnosed with PD, HSMI, CMS and PGI in addition to autumn disease. The material was collected at the Veterinary Institute in Bergen from the years 2005, 2008 and 2009, and from the Veterinary Institute in Harstad year 2009. The main purpose of the study was to examine the presence of *D. lepeophtherii* in the diagnostic material and to evaluate a possible association between the microsporidian and any of these diseases.

The results of the study showed no association between *D. lepeophtherii* and the diseases PD, HSMI and CMS. The results showed, however, an increased *D. lepeophtherii* burden in PGI samples and in the 2008 autumn disease individuals in material from Southern Norway. The results therefore indicate a possible association for *D. lepeophtherii* to the conditions autumn disease and PGI in farmed salmon from Southern Norway. In the material from Northern Norway the *D. lepeophtherii* burden was very low. In this material *D. lepeophtherii* was not associated with any disease condition.

The study also showed that stored formalin fixed paraffin embedded material is very adequate for this type of work. The Norwegian Veterinary Institute has a large amount of historical diagnostic material that can be used for further studies on different disease conditions.

Introduction

The “autumn disease”

During fall 2008, the Norwegian Veterinary Institute (NVI) received several diagnostic samples of Atlantic salmon (*Salmo salar*) with shared diagnostic features. The samples were investigated for bacterial or viral causative agents with negative result. Altogether there were cases from 25 locations with similar clinical, macro- and histopathological findings. The locations were scattered from Rogaland to Møre and Romsdal. Up to 20 per cent mortalities in addition to reduced growth were registered at some locations.

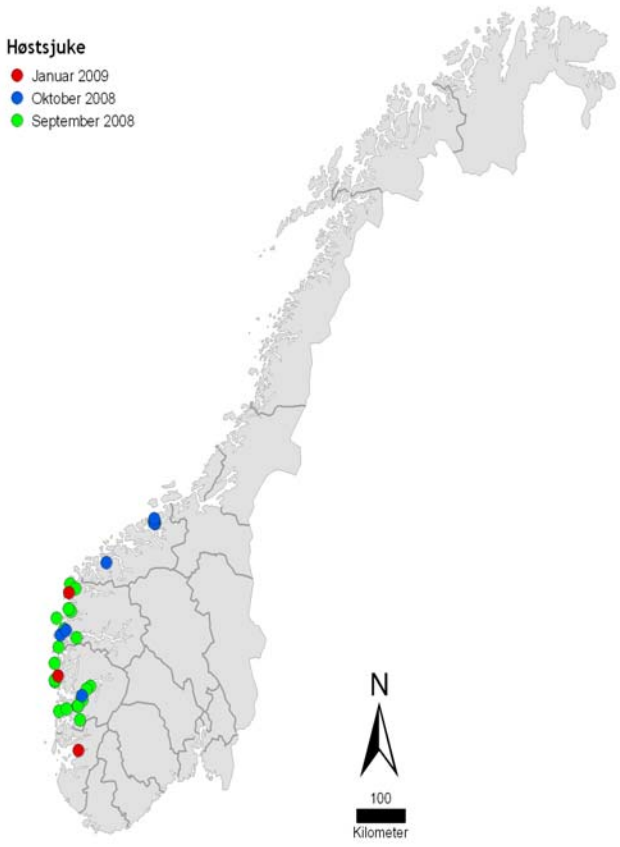


Figure 1 - Map showing locations with diagnosed autumn disease in September 2008 (green), October 2008 (blue), and January 2009 (red). Photo: Epidemiological unit, The Norwegian Veterinary Institute.

Clinical signs commonly reported for affected salmon were respiratory problems. Predominant autopsy findings were usually dilated operculum, swollen and pale gills, ascites, yellowish liver, swollen, dark spleen, and petecchial bleedings in perivisceral adipose tissue. Generally the fish also had lack of gut contents. Histological examination revealed epithelial proliferation, necroses and circulatory disturbances in the gills. Some fish also had renal interstitial bleeding, inflammation cell infiltration, hyperplasia of Bowman's capsule, peritonitis, hemosiderosis and steatitis.

Due to unknown cause of the condition and epidemic occurrence during autumn, the disease was called "autumn disease" [72]. Recent studies indicate that a microsporidian hyperparasite, *Desmozoon lepeophtherii* [21] (also called *Paranucleospora theridion)* [56], may be associated with "autumn disease". Recently it has been suggested that this microsporidian parasite is connected with other diseases that occur in the farmed Atlantic salmon industry [50,51,53,56].The diseases suggested associated with *D. lepeophtherii* are pancreas disease (PD), proliferative gill inflammation (PGI), cardiomyopathy syndrome (CMS), and heart and skeletal muscle inflammation (HSMI).

General features of the microsporidia

The microsporidia were discovered 150 years ago. The first microsporidium described was *Nosema bomicis,* a parasite of silkworms [48]. Sprague *et al*., 1992, described in their report more than 1200 species in phylum *Microsporidia* [68]. Later on several additional species have been added. Microsporidians are best known as parasites of insects, but they are also responsible for infections in crustaceans, fish and mammals. The first disease caused by a microsporidian parasite recognized in vertebrates was infection by *Glugea anomala* in threespined sticklebacks (*Gasterosteus aculeatus*) [40]. There are more than 156 microsporidian species distributed in 14 genera [41] infecting fish. The end stage of the microsporidian life cycle is the creation of mature spores. The spore stage has several structures characteristic for the microsporidia, and is very suited for transmission.

The microsporidian life cycle

Microsporidia are fungi like, eukaryotic, obligate intracellular, spore forming parasites. They parasitize on a wide range of both vertebrate and invertebrate hosts, including humans. They lack mitochondria [26], and are dependent on one or several hosts to reproduce and carry out their lifecycle. Microsporidia were earlier suggested as primitive eukaryotes, but recent studies have shown that they are highly specialized eukaryotes that have gone through a reductive development for adaptation to an obligate intracellular life [31]. The microsporidia are now classified as fungi [75].

Microsporidian life cycles and transmission strategies are known as some of the most complex known among parasites. The strategies include use of direct and indirect life cycles, with both horizontal and vertical transmission [14]. The general transmission route is, however, horizontal transmission. The parasite can be transmitted sexually, orally or by direct invasion through the host epithelium. Infective spores enter the host and may further on spread to host target tissue and cells [13]. Microsporidia produce highly environmentally resistant spores with unique ultra-structural characters, which can survive outside the host cell for a long period of time (Fig. 2).

There are three distinct phases in the development of microsporidia.

1. The environmental infective phase with the mature spore.
2. The proliferative phase with merogeny that accumulate number of spores.
3. The sporogonic phase with sporogeny. Sporonts produce sporoblasts that further develop into mature spores [5].

The spore size varies from 1 to 40µm, and the shape in most species is oval or pyriform, but other shapes are not unusual [73]. The two major parts of the spore wall is the exospore and the endospore. The external proteinaceous electron dense layer, the exospore, is about 10 – 200 nm thick depending on the genera [39]. The endospore layer consists mainly of chitin and protein layers below the outer exospore, and can be up to 100 nm thick [15,73]. The spore wall is also the site of active and passive transport of solutions, and acts as a pressure vessel during germination [24]. A cell membrane separate the spore wall from the spore cytoplasm, also called sporoplasm. This contains either one individual nucleus (monokaryon) or two associated nuclei (diplokaryon) [73]. The cytoplasm possesses free ribosomes of a prokaryotic type with a

70S unit that dissociates into 50S and 30S subunits containing 23S RNA and 16S RNA, respectively, and they lack a separate 5.8S RNA [12,29,76]. The unique infectious apparatous: the polaroplast, the polar filament (or the polar tube), and the posterior vacuole are the three fundamental structures that are related to microsporidian infection. The polaroplast lies in the anterior region of the spore and contains several lamellar membranes. A hollow structure known as the polar tube; is connected to the top of the spore by an anchoring disc. This tube is arranged in helical coils down from the anchoring disk to the posterior part of the spore. Number of coils vary among the different species, and the tube vary in length from 50-500µm [32]. During germination, the polar tube turns inside out of the spore, and forms a canal by which the infective sporoplasm can reach new host cells. At the posterior part of the spore, there is a clear membrane bound area, also called the posterior vacuole. This is a membrane-bound organelle which swells just before germination [5]. It is suggested that chemical stimulus such as pH and cations in the host trigger the coiled polar tube to elongate and germinate [25,71], forming a hollow tube that can infect the host cell. The posterior vacuole expands during the germination and pushes the infective spore content into the polar tube and further into the host cell cytoplasm.[73]. After the discharge of the sporoplasm, only the spore wall, plasma membrane, and some membrane profiles remain in the previous spore, forming a "spore ghost" [78].

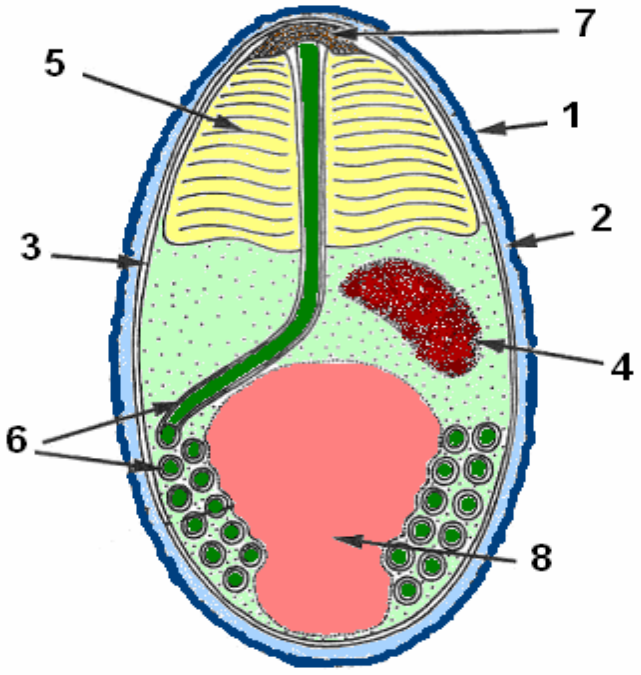


Figure 2 - Diagram of a generalized microsporidian spore. Spore wall containing three layers: **1** exospore, **2** endospore and **3** inner plasma membrane, **4** monokaryon nucleus (some microsporidia have diplokarya nuclei), **5** polaroplast, **6** polar tube, **7** anchoring disk and **8** posterior vacuole. (Figure modified from figure in chapter 3; kompendium i fiskesydommer") [30].

Once the sporoplasm reaches the host cell cytoplasm, the parasite is referred

to as a meront. This is the proliferative phase (merogeny). These cells have a round shape and are surrounded by a unit membrane [73]. The meront cytoplasm contains either one single nucleus or a double nucleus depending on the species. The cytoplasm also contains a poorly developed endoplasmatic reticulum and many free ribosomes [6]. Microsporidia can divide in three different ways: either binary fission, plasmotomy or multiple budding.

The ultrastructural evidence that the parasite has entered the final sporoginal phase of reproduction is when an electron dense material are deposited on the outer plasmamembran of the meront [5,73]. They are now classified as sporonts, the initial stage of sporogeny. Sporonts undergo further division, and become sporoblasts, the products of the last cell division. This is the last stage of the intracellular host cell dependent development, resulting in fully matured microsporidian spores [11].

Host- parasite relationships of fish microsporidia can be divided into two different groups [67]. The one that does not form xenoma (e.g., *Nucleospora* and *Pleistophora*), and those forming xenoma (e.g., *Glugea, Loma* and *Tetramicra*). The term "xenoma" represents a highly hypertrophic host cell that contains multiple spores and developmental stages of the microsporidian. The hypertrophied cells can develop into large, white macroscopically visible cysts, measuring up to 500 µm [43,62].

Desmozoon lepeophtherii

Mark Freeman discovered in 2002 a previously unknown hyperparasitic microsporidium infecting the salmon louse *Lepeopthteirus salmonis* [19]. The main goal of his study was to detect any *L. salmonis* specific pathogens with a potential as biological control agents against the salmon louse. He sampled sea lice from 15 farm sites in Scotland, and discovered lice heavily infected with an unidentified microsporidium. Using transmission electron microscopy (TEM) he discovered cysts containing spore-like structures distributed within the haemocoel of the salmon lice. TEM results showed the infection in the louse to be associated with epidermal cells beneath the cuticula (Fig. 3). Based on rDNA Freeman placed the discovered microsporidianum in the phylogenetic clade *Enterocytozoonidae* with the genera *Nucleospora* and *Enterocytozoon* [20]. Based on target cells (desmocytes) and host name the microsporidium was given the name *Desmozoon lepeophtherii* [21]. As part of the work to

characterize the life cycle of *D. lepeophtherii* Atlantic salmon was screened for the microsporidium. Using PCR Freeman found salmon testing positive for the parasite[19]. DNA that was isolated from infected salmon lice and positive Atlantic salmon, where compared, and were found to come from the same species. He therefore suggested the microsporidium to be transmitted between Atlantic salmon and sea louse. The life cycle was later further described by Nylund *et al*., after identifying it in Norwegian Atlantic salmon and in salmon louse (*L. salmonis)* in 2008. It was then named *Paranucleospora theridion* by Nylund *et al*., 2010 [56]. Molecular rDNA studies have found the rDNA small subunit to be identical between the Scottish and Norwegian isolates, suggesting them to be the same species (SSU; GenBank accession no: AJ 431366) [55]. *D. lepeophtherii* has also been found in the sea louse (*Caligus elongatus*), in sea trout (*Salmo trutta*) and in rainbow trout (*Oncorhynchus mykiss*) [56]. From the ultrastructural and development examinations performed in Norway by Nylund et *al*., 2010 [56], it is proposed that the microsporidium has a very complex life cycle (Fig 4). The Atlantic salmon acts in this case as the intermediate host, while the copepod *L. salmonis* is defined as the main host. Real time PCR results have shown the microsporidium to be present in all developmental stages of *L. salmonis* [56] and *C. elongatus.* The development of the microsporidium in salmon occurs in two different cycles. The first cycle occurs in the cytoplasm of several cell types. These cells include macrophage like cells, blood vessel endothelial cells, polymorphonucleate leucocytes in the host tissue and also the gill epithelial cells and skin cells [56]. Spores developed in this first cycle are approximately 0,8-1,8 µm in diameter, have a thin spore wall and a short polar tube, and are referred to as auto infective spores. In development cycle two there is a production of ellipsoidal environmental spores with a thick endospore wall and thin exospore wall. The polar tube from this spore is longer than the polar tube found in the auto infective spores, and is only found within the nucleus of the epithelial cells of the fish gill and skin. Mature spore size is about 2,4 -2,7 µm long × 2-0 - 2,1 µm wide [56]. It is shown that the infected cells contain one or more spores in each nucleus.

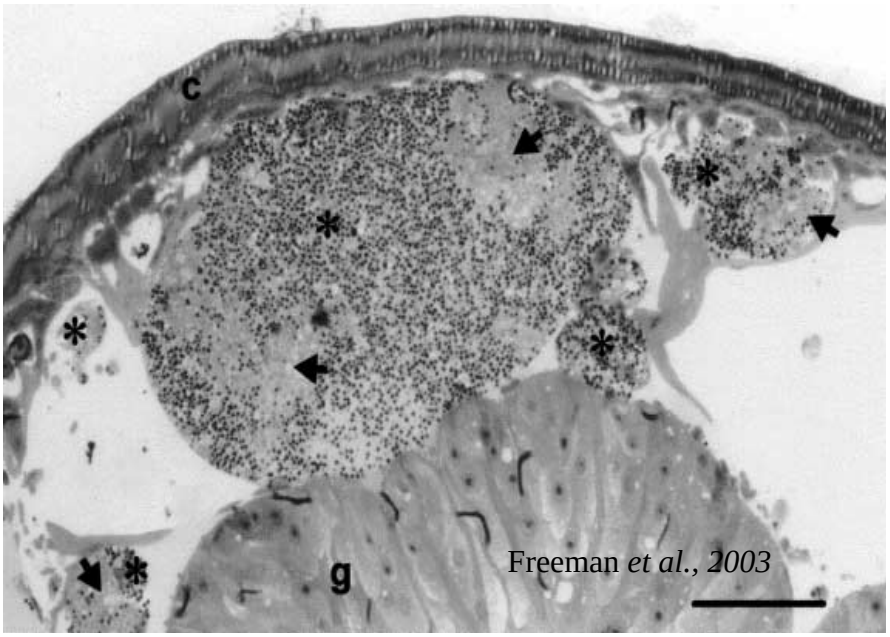


Figure 3 -Transverse semi-thin section through the abdomen of an infected adult female of *L. salmonis.* Xenoma (*) developing between the cuticle (c) and the gut (g). (Bar= 100 µm).

Salmon lice that feed on an infected fish receive the microsporidian spores through its ingestion of fish mucus, blood and epithelial cells. This is assumed to be the transmission route between the intermediate host and main host. The development within *L. salmonis* results in two different types of spores, macro- and microspores. The purpose of the macrospores is so far unknown. Microspores are spherical. Infection by the microsporidium occurs in the glycocalyx border that lies beneath the cuticle. Cells often infected are epithelial cells, connective tissue, desmocytes, satellite cells and haemocytes. Spore development is in direct contact with the cell cytoplasma the entire time of the development cycle. Growth of the parasite within these cells results in massive host cell hyperthrophy and large xenoma-like cysts that forms directly under the cuticle (Fig. 3) which contains large numbers of different development stages of *D. lepeophtherii* [20,56]. Spores can then be released from these into the sea water. Free spores are spread horizontally through the water. Infection of the salmonid host is believed to occur through the gills.

Lifecycle of *Desmozoon lepeophtherii*

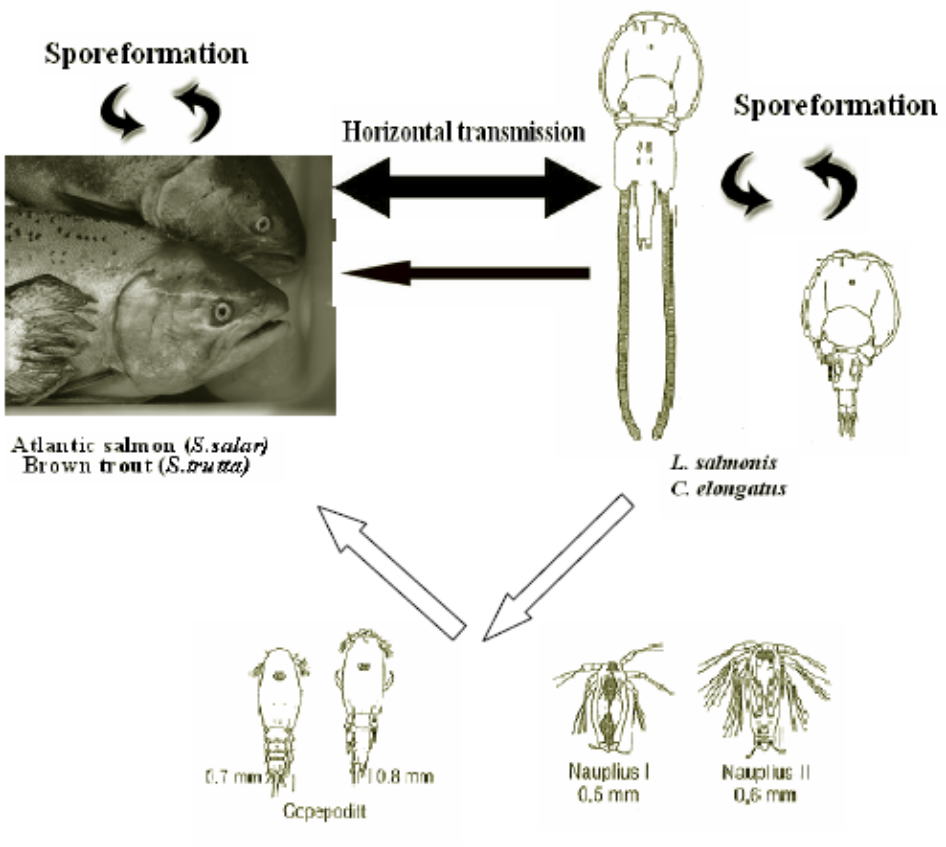


Figure 4 –Lifecycle of *D. lepeophtheri.* Spore formation occurs in both salmon lice and salmonids. Mature spores are taken up through the fish gills. In salmon two types of spores develop (autoinfective spores and environmental spores). These spores are trensferred to salmon lice when they eat the skin epithelial cells and blood of the salmon. Spore formation in salmon lice occurs directly beneath the cuticle, and the spores are spread further to the water. (Figures obtained and modified from www.genok.no and Schram 1993) [65].

Pancreas disease (PD)

Pancreas disease (PD) is a severe viral fish disease with major influence in salmonid fish farms and aquaculture. PD was first detected and described in Scotland in 1976 [46]. The disease has later been described in countries like Ireland [47] and Norway [60]. In 1995 an alphavirus (Salmonid alphavirus or SAV) was reported to be the causative agent of the disease [49]. Clinical signs of PD are often sudden drop of appetite and abnormal swimming behaviour near the water surface. The disease leads to great economical losses in the industry due to both mortality and reduced growth. Reduced growth is associated with pancreatic

tissue damage [46,70]. Histopathological findings in affected fish are necrosis and loss of exocrine pancreas, necrosis and inflammation in heart muscle ventricle and inflammation and degeneration in red and white skeletal muscle [44,70]. The causative virus of PD is divided into six subtypes (SAV 1, 2, 3, 4, 5 and 6) based on phylogenetic studies [22]. The subtypes show geographical separations in Europe, and SAV3 (Norwegian salmonid alphavirus, NSAV) is the only type seen in Norway [28]. Since the mid-1990s PD has been diagnosed yearly in Norway. The number of recorded cases was low in the period 1998-2002, but increased drastically until 2008 [7] with the number of affected locations remaining high. The diagnostic criteria for PD are based on histological examinations and detection of virus.

Heart and skeletal muscle inflammation (HSMI)

A new disease was discovered in farmed Atlantic salmon in Norway in1999 [36]. The disease was also reported from the United Kingdom [16]. Several farming sites experienced fish mortality of unknown character. Autopsy findings of the fish point out ascites, yellowish liver, swollen spleen and petechiae in the perivisceral fatty tissue [36].Tests performed showed that the most common findings was pale heart due to severe epi- , endo- and myocarditis [33,36]. The infested fish showed severe inflammation of the red skeletal muscle. Based on the gross and histopathological findings the disease was called heart and skeletal muscle inflammation (HSMI). The HSMI related lesions may histopathologicaly resemble lesions found in fish with cardiomyopathy syndrome (CMS) [17] and pancreas disease (PD) [18,45]. However, HSMI stands clearly out from these diseases. In HSMI the pancreas is not affected, and the affected compact layer of the heart found in cases with HSMI is rarely observed in cases with CMS [33]. To investigate the cause and pathogenesis of HSMI, an experimental study was performed in 2004. The study proved through cohabitation and by injection that the disease is transmissible[34]. Furthermore, an experimental transmission study [35] indicated that the casual agent is a virus. TEM studies of the disease have observed virus-like particles [77]. Recent studies have found a new reovirus, with the suggested name Piscine reovirus (PRV), to be associated with HSMI. This virus is overrepresented in fish with HSMI and is also found to be associated with HSMI lesions [59]. The diagnostic criteria for HSMI are based on histological examinations.

Cardiomyopathy syndrome (CMS)

Cardiomyopathy syndrome (CMS) is a cardiac disease in farmed salmon. It was first discovered in Norway in the mid-1980s [1,17]. Later on the disease has been detected in the Faroe Islands, Scottland and Canada [8,9,63]. Generally farmed Atlantic salmon in sea cages are affected, but the condition is also seen in wild salmon[61]. CMS appears 14 to 18 months after transfer to seawater [17], and affects large fish that are ready for slaughter. Pathological findings indicate that CMS is a chronic disease that develops over a long period of time. Histopathological changes are found in the spongious myocardium of the atrium and ventricle. Typical findings are inflammatory cell infiltration, and proliferation of the endocardium[9,17,23]. An experimental transmission study done by Bruno *et al.,* 2009 [9] demonstrated that CMS could be reproduced in *naïve* Atlantic salmon by injecting tissue from affected fish. Epidemiological studies have previously indicated that CMS has an infectious causative agent [10,23]. Recent publications support this statement. A novel piscine totivirus, named Piscine myocarditis virus (PMCV), is shown to be associated with the development of the disease [42]. The diagnostic criteria for CMS are based on histological examinations.

Priloferative gill inflammation (PGI)

To diagnose gill related fish diseases is often challenging. The organ is exposed to the environment, and therefore the factors causing irritation may be numerous. The fish gills have relative few cell types. Different ethiology may therefore be manifested with similar histological changes. Several studies have suggested a multifactorial etiology for gills described with proliferative gill inflammation. Causative factors may also be multiple enforcing each other. Gill damage may be induced by chemical and mechanical irritants like algae and jellyfish, poor water quality, opportunistic pathogens and primary pathogens (bacteria, uni- or multicellular parasites and fungi, viruses or amoeba). Atlantic salmon paramyxovirus (ASPV) has been suggested to take part in the development of PGI [37,38], the same with salmon gill poxvirus (SGPV) [54]. Epitheliocysts caused by bacteria is also a typical finding in fish with PGI [52]. *Ichthyobodo* sp. parasites have been seen in fish with PGI-like lesions. Recent studies have indicated that *D. lepeoptherii* can be related to PGI [21,51,69]. PGI is a condition that has been known to farmed Atlantic salmon in Norway since the mid-1980's.

Included in the classification of gills suffering from proliferative gill inflammation are four features; inflammation, cell death, epithelial hyperplasia and circulatory disturbances [38]. The diagnostic criteria for PGI are based on histological examinations.

Aims for the study

There are two main goals for the study.

1. Main goal one is to examine official diagnosed material for the presence of the microsporidian parasite *D. lepeophtherii* using Real time RT-PCR.
2. Main goal two is to look for a possible relationship between *D. lepeophtherii* and the official diagnosis PD, HSMI, CMS, PGI and autumn disease.

Subgoal for the study is to establish the use of formalin fixed paraffin embedded material for use in nucleic acid based research.

Materials

In this thesis, (comma) is used as decimal separator as used in Norwegian orthography. As part of routine inspections and in connection to disease outbreaks formalin fixed tissue material from fish in different cultivation locations is sent to NVI. The material is then paraffin embedded and examined for possible diagnosis by histopathological methods. All paraffin embedded material is thereafter stored. Information of all material submitted to the NVI is stored in the sample journal system. The sample information is therefore retrievable by database searches.

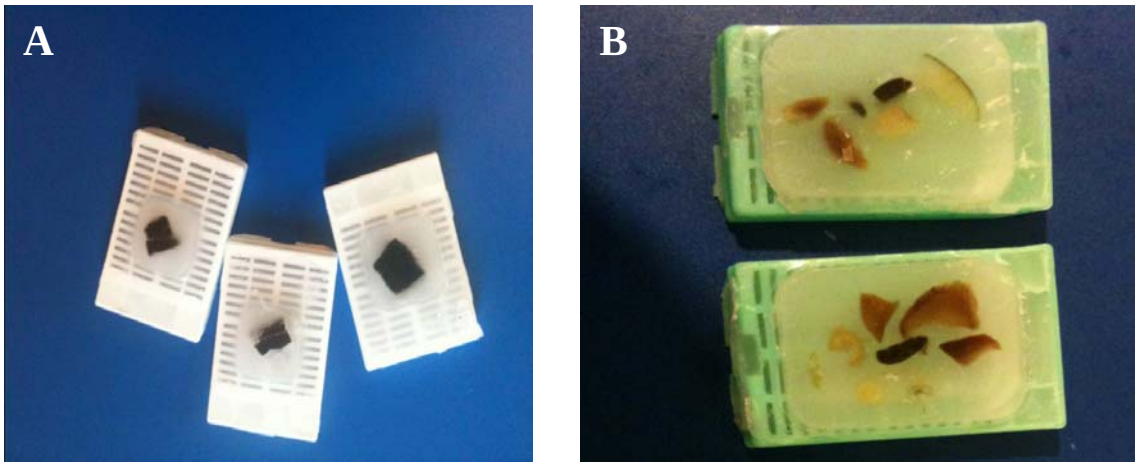


Figure 5 - Formalin fixed paraffin embedded tissue material (A), single organ (renal tissue) blocks NVIB, (B) multiorgan blocks NVIH

This study will be based on paraffin embedded material with officially set diagnosis, PD, CMS, HSMB and PGI. In addition material with “autumn disease” will also be used. The latter is as mentioned above, not an official diagnosis. The material was gathered from year 2005, 2008 and 2009 at the Norwegian Veterinary Institute Bergen (NVIB) and 2009 from the Norwegian veterinary institute in Harstad (NVIH). The year 2005 was chosen to include PD material before the common use of an anti SAV vaccine. This is also before the autumn disease outbreaks in 2008. The year 2009 was chosen to get PD material from after the introduction of a PD vaccine and after the 2008 autumn disease outbreaks. To ensure that the fish had a systemic microsporidian infection renal tissue was chosen. As far as possible each diagnosis was represented by 4 individuals from 10 cases for each year. Low outcome samples were re-purified once. If the yield was still low the sample was discharged as low

quality. Altogether total RNA was purified from 498 individuals representing 135 diagnostic cases (Table 1). From these were a total of 468 individuals from 133 cases examined with the use of real time RT-PCR (Table 2)

PURIFIED MATERIAL, number of individual samples

Table 1 - Cases and individual samples purified. NVIB and NVIH 2005, 2008 and 2009

DISEASE	YEAR	CASES	INDIVIDUALS
PD (VIB)	2005	10	40
PD (VIB)	2008	10	40
PD (VIB)	2009	10	40
PD (VIH)	2009	2	30
HSMI (VIB)	2005	9	35
HSMI (VIB)	2008	8	31
HSMI (VIB)	2009	10	40
HSMI (VIH)	2009	11	42
PGI (VIB)	2005	9	24
PGI (VIB)	2008	7	27
PGI (VIB)	2009	8	27
PGI (VIH)	2009	6	15
CMS (VIB)	2005	9	27
CMS (VIB)	2008	3	9
CMS (VIB)	2009	6	16
CMS (VIH)	2009	10	27
"AUTUMN DISEASE"	2008	7	28
TOTAL NUMBER		**135**	**498**

PCR MATERIAL, number of individual samples

Table 2 - PCR material from cases and individual samples. NVIB and NVIH 2005, 2008 and 2009

DISEASE	YEAR	CASES	INDIVIDUALS
PD (VIB)	2005	10	38
PD (VIB)	2008	10	38
PD (VIB)	2009	9	36
PD (VIH)	2009	1	16
HSMI (VIB)	2005	9	35
HSMI (VIB)	2008	8	30
HSMI (VIB)	2009	10	38
HSMI (VIH)	2009	11	42
PGI (VIB)	2005	9	23
PGI (VIB)	2008	7	27
PGI (VIB)	2009	7	25
PGI (VIH)	2009	6	14
CMS (VIB)	2005	9	26
CMS (VIB)	2008	3	9
CMS (VIB)	2009	6	16
CMS (VIH)	2009	10	27
"AUTUMN DISEASE"	2008	7	28
TOTAL NUMBER		**133**	**468**

Methods

RNA purification

The purification of total RNA from formalin fixed paraffin embedded tissue sections was performed by using a specially designed kit (RNeasy®FFPE) from QIAGEN®.

The method is performed according to the Qiagen user manual from January 2006 and September 2010. Steps involving xylene treatment were performed in a ventilation hood. By isolating RNA longer than 70 nucleotides, the kit provides restoration of usable RNA fragments suitable for real time RT- PCR. Due to embedding conditions and fixation, nucleic acids in paraffin embedded material, may be strong fragmented and chemically modified by formaldehyde. This kit is optimized to reverse the impact of formaldehyde without additional RNA degeneration. RNeasy FFPE also selectively removes double-stranded DNA from the material. Total RNA is purified from the sections due to specially optimized lysis conditions.

General principles for the purification are as follows: Tissue sections that are freshly sliced are deparaffinized with xylene. The sections are thereafter washed with 100 % ethanol to remove the xylene. Further the samples are incubated with buffer PKD and proteinase K (both part of the RNeasy FFPE kit), an optimized lysis buffer that release RNA from the sections. Next the samples are incubated in higher temperatures for 2×15 minutes, first at 56ºC, and then at 80ºC. The higher temperatures reverse formalin cross linking of the nucleic acids, improving the RNA yield and sample quality. The samples were DNase treated (buffer and DNase part of the RNeasy FFPE kit). This handling is optimized to remove all genomic DNA, also including some small DNA fragments which often are present in FFPE samples after formalin fixation and long storage periods. The samples are then mixed with Buffer RBC (part of the RNeasy FFPE kit) and ethanol. The ethanol is added because it supplies an appropriate binding condition for RNA to the matrix in specialized RNeasy Mini Elute spin columns. RNA bound to the column matrix was then washed with buffer RPE (part of the RNeasy FFPE kit) to remove non RNA material. The columns were centrifuged and allowed to dry for 5 minutes to remove all RPE buffer. It is important that the columns are dry, because residual ethanol may interfere with downstream reactions. The last step of the procedure is to elute the RNA with 30 µl RNase-free water that is placed directly on the spin

column membrane. After centrifugation on full speed for 1 minute the total RNA is collected in the bottom of the centrifugation tube. Pureness and concentration of the eluted RNA was determined with a Nanodrop 2000 spectrophometer. The spectrophometer measures RNA nucleic acid concentration (NAC) by measuring 260 nm absorbance (A_{260}), concentrations and sample purity (260/280 nm ratio). RNA samples were stored at -80ºC until they are analyzed by real time RT-PCR.

Real time PCR

Polymerase chain reaction (PCR) allows for specific detection of minor amounts of a target DNA sequence by amplification of the sequence. Specificity of the reaction is ensured through the use of double thread dependent DNA polymerase and specific primers. Pre amplification reverse transcription from RNA to DNA with the use of reverse transcriptase makes it possible to also detect RNA sequences (reverse transcriptase PCR or RT-PCR). The process is well known and I will therefore not describe the amplification cycle in detail here. Real time PCR is a further development of the PCR technique. In real time PCR a probe with a fluorescing reporter and a quencher inhibiting the reporter will anneal to the targeted sequence between the primers. The Quencher is more electronegative than the reporter and will inhibit excitation of reporter electrons. The DNA polymerase used for the amplification has an exonuclease activity and will therefore fragmentize the probe separating the reporter and the quencher. This allows for excitation of the reporter and thereby a fluorescing signal. Each successful amplification will yield a fluorescence signal that will be detected by the real time PCR machine. This makes it possible to detect the outcome of each amplification cycle and not just the end product as in conventional PCR. Therefore the name real time PCR. This makes real time PCR useful for quantitative detection. Results from real time PCR analyzes are given as cycle of threshold values (Ct). This is the cycle when the machine starts to detect linear amplification results i.e. product doubling per cycle.

Real time PCR was performed using OneStep RT-PCR kit from QIAGEN®. The real time RT-PCR amplification was performed on a Stratagene Mx 3005P QPCR System. Sequence for primers and probe specific for *D. lepeophtherii* (NUC), was as described by Nylund et *al*., 2010 [56]. Primer probe concentrations for the assay were 600 nM for each primer and 175 nM

for the probe. Quality of the purified RNA material was assured by the use of the house-keeping gene elongation factor 1 alpha (EF1A) [58]. Primer probe concentrations for this essay were 900 nM for each primer and 200 nM for the probe. Template amount used was calculated based on RNA concentration outcome from the purification. The template quantity vitiated from 1 µl to 12, 5 µl (see appendix 1). The OneStep RT-PCR reaction mastermix was prepared with 4 µl RT-PCR buffer, 0.3 µl ROX, 0.8 µl dNTP, 0,2 µl NUC F, 0,2 µl NUC R, 0,2 µl NUC probe (MGB), 1 µl MgCl2, 0,1 µl RNaseOut and 0,8 µl RT-PCR enzyme mix for each PCR well with a total mastermix of 7,6 µl. Volume was adjusted by adding water to a total of 20 µl reaction volume per sample. A sample previously diagnosed as autumn disease was found to have a high amount of *D. lepeophtherii*. RNA from this sample was diluted to give a Ct-value of 21 and used as a positive control in all assays throughout the study. A non template control (NTC) was also used in all assays. This was to investigate any possible contaminations. The real time RT-PCR analyses were performed by a Stratagene Mx 3005P QPCR System in these terms: Reverse transcription: 30 min at 50ºC. Initial PCR activation step: 15 min at 95ºC and 45 cycles of: denaturation; 30 sec at 94ºC, annealing; 45 sec at 55ºC and extension; 45 sec at 72ºC. In samples that were found to be negative, Ct-value was set to be 45 for easier evaluation for the results. As mentioned above elongating factor 1a (EF1A) was also amplied for each sample. This is a housekeeping gene used as endogen reference in salmon samples. Stable successful amplification of EF1A ensures both the quality of the purified RNA and the RT-PCR reaction. The analysis controls that the purification and PCR reaction have been successful. The quantity of the housekeeping gene determines the sample quality. High EF1A Ct-value indicates low template quality as long as the amount template added to the reaction is stable. These samples should be rejected. In cases where the EF1A Ct-value diverged by more than 3 from the mode Ct-value of the total amplified group, the sample was rejected.

A cut off value was estimated by making a two fold dilution serial of the positive control with three parallels. A real time RT-PCR analysis was performed for the detection of *D. lepeophtherii.* Cut off value was set as the cycle where one or more reactions were negative.

Table 3 - Primers and probes used for real time RT-PCR

Primer / probe	Sequence	Bp	Position	Reference
Nuc- fwd Nuc- rev Nuc- probe	5`-CGGACAGGGAGCATGGTATAG-`3 5`-GGTCCAGGTTGGGTCTTGAG-`3 5`-TTGGCGAAGAATGAAA-3`	59	522-542 580-561 544-559	Nylund *et al.*, 2010
$EF1A_A$-fwd $EF1A_A$-rev $EF1A_A$-probe	5`-CCCCTCCAGGACGTTTACAAA-`3 5`-CACACGGCCCACAGGTACA-3 5`-ATCGGTGGTATTGGAAC-`3	57	800-820 839-857 821-837	Olsvik *et al.*, 2005

Calcofluor white stain

This method detects organisms which contain chitin. Calcofluor (or bleach) binds to cellulose or chitin in the cell wall of fungi and microsporidia. A positive binding will fluoresce with green or blue colours when exposed toUV-light with the correct wavelength (optimal wavelength is 347 nm[27]). Morphology of fluorescing structures was used to determine the probability of the structure being a microsporidian spore.

Kidney tissue from individuals with a *D. lepeophtherii* Ct-value similar to the mean value for each group (diagnosis, year and laboratory of origin) was chosen for calcofluor staining (Table 4). Only samples obtained from NVIB were used for calcofluor staining. Three µm tissue sections were placed on poly L-lysine coated slides. The thickness of the uncoloured sections was set to 3µm. The sections were deparaffinzed in xylene and rehydrated in ethanol (dilution series of 100, 96, 70, and 50 %, 3 minutes in each). The preparations were then encircled with a PAP-pen. After drying, the sections were added 1 drop of KOH (15%) and 1 drop of calcofluor (FLUKA ®analytical 100 ml, cat. nr 18909). After 1 minute incubation, the samples were covered with a coverslip and microscoped with epifluorescense, 347 nm. The fluorescence microscopy was performed using a Nikon TE2000 microscope.

Table 4- Samples selected for the calcofluor white stain method.

PD		HSMI		CMS		PGI		AUTUMN DISEASE	
Samples	**Ct-values**	**Samples**	**Ct-values**	**Samples**	**Ct-values**	**Samples**	**Ct-values**	**Samples**	**Ct-values**
F11/08-7	33,45	F417/08 F474/08 F200/09 F150/09	26,81 26,82 37,55 37,69	F24/08	32,98	F343/08 F449/09 F61/09	21,57 24,48 28,68	F419/08 F379/08	15,79 22,87

Results

Purification of paraffin embedded material

Outcome of the RNA purification varied from 0 to 1000 ng/µl. Samples with low RNA yield showed correspondingly poor 260/280-ratio, and were disqualified from further use in the project. Submitted cassettes from each case had dissimilar amount of kidney tissue, and this was taken into account. If the tissue sample was too small, the number of cut sections was increased to get a sufficient amount of tissue.

The purified materials submitted from the NVIH were multiorgan cassettes. Empirical observation from the tissue material was that the amount of sliced section could be different compared to the sections from the NVIB. Still, the results proved to function and there were no found differences on the purified samples and RNA concentrations from the two institutes.

Real time RT-PCR results

The results from the realtime RT-PCR analysis for the detection of *D. lepeophtherii* are given as average cycle-threshold value (Ct-value) ± standard deviation.
Cut off value for the *D. lepeophtherii* assay was found to be Ct 38. Standard curve slope for the essay was -3,11. Efficiency E was 1,1.
The results from the housekeeping gene give an indication of the quality of the purified RNA and are analyzed at the same time as the PCR for detecting *D. lepeophtherii.* As indicated in Figure 6 most EF1A Ct-values from each group clustered together. This indicated that the RNA in the material was intact and comparable. There was now difference in sample quality in between the different year groups.

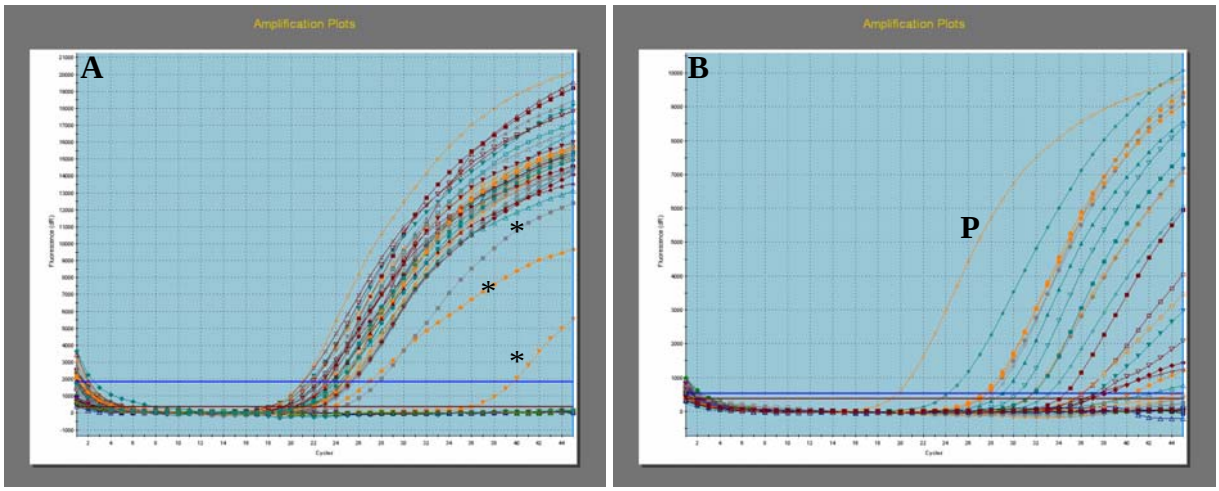


Figure 6 - Results of real time RT-PCR Ct-values for HSMI 2009 NVIB. (A) Amplification plots with primer probe specific for housekeeping gene (EF1A). (B) Amplification plots with primer probe specific for *D. lepeophtherii*. Diverging EF1A curves represent samples which are rejected (*), positive control (P).

Pancreas disease

Desmozoon lepeophtherii Ct- values for 2005, 2008 and 2009 PD samples (NVIB).

Average real time RT-PCR results for PD cases at NVIB for 2005 were 41,3±5,2, for 2008 36,0 ± 7,1 and for 2009 38,7 ± 5,8 (all values given as mean Ct-value ± standard deviation) (Fig. 7). Thirty-eight samples were originally analyzed for the detection of the parasite in cases with pancreas disease in 2005. Results from the amplification plots of housekeeping gene eliminated 7 samples that could not be taken into consideration due to low quality. Five of 10 diagnostic cases distributed from March to December were detected positive for *D. lepeophtherii*. Out of 31 individuals examined, 12 fish proved positive for *D. lepeophtherii*. From the year 2008, all samples analyzed had sufficient RNA quality. Nine out of 10 diagnostic cases had positive individuals and 27 out of 38 individuals were positive for *D. lepeophtherii*. Also all the analyzed material from individuals with the diagnosed pancreas disease in 2009 could be used. Eight out of 9 diagnostic cases had individuals positive for *D. lepeophtherii* and 23 out of 36 individuals tested positive for *D. lepeophtherii*. Numbers are shown in Table 4.

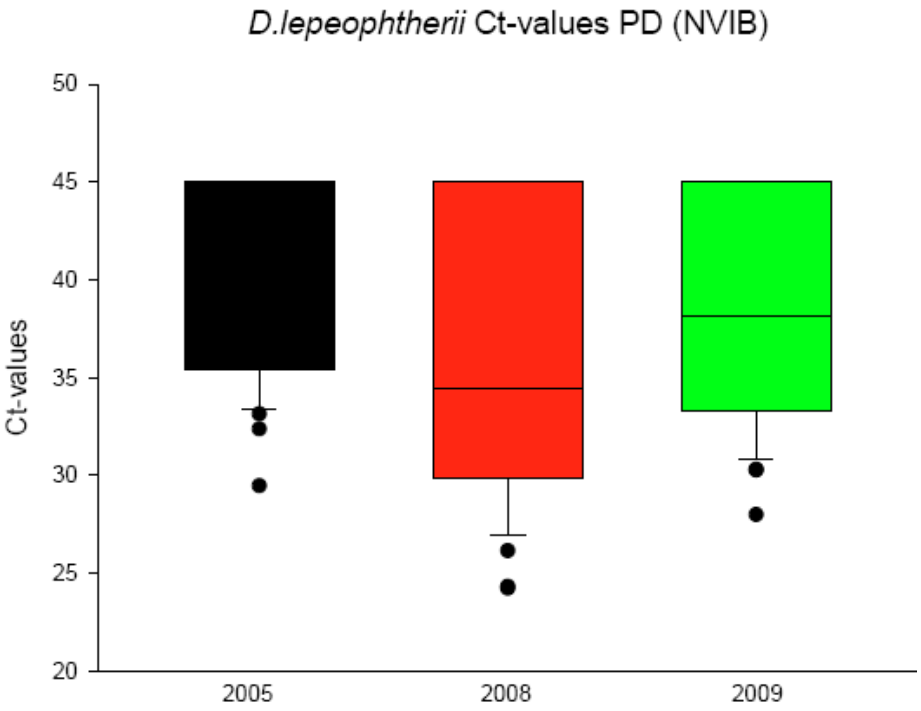


Figure 7- Real time RT-PCR results for *D. Lepeophtherii* specific analysis of PD diagnosed material from NVIB year 2005, 2008 and 2009. Results shown as Ct-values

Table 4 - Displays an overview of PD diagnosed cases and samples from the Veterinary Institute Bergen (NVIB) investigated for *D. lepeophtherii*..

Year	Total cases	Individual samples	Samples rejected due to low quality	Positive samples
2005	10	38	7	12
2008	10	38	0	27
2009	9	36	0	23

Heart and skeletal muscle inflammation

D. lepeophtherii Ct- values for 2005, 2008 and 2009 HSMI samples (NVIB).

Average real time RT-PCR result for HSMI Bergen 2005 was 37,4 ± 6,2, for 2008 27,2 ± 6,9 and for 2009 38,4 ± 7,1 (all values given as mean Ct-value ± standard deviation) (Fig. 8). A total of 35 individual samples from 9 cases diagnosed with heart and skeletal muscle inflammation (HSMI) were examined for the presence of the microsporidian parasite in 2005. All cases had one or more fish that showed positive Ct-values. Twenty-five out of 35 individual samples were found positive for *D. lepeophtherii.* From 2008 30 samples were examined from 8 cases. Thirty out of 30 samples were found positive for *D. lepeophtherii.* From the year 2009, a total of 38 samples were examined. Three of these samples were rejected due to low quality RNA. Nineteen out of 38 samples were found positive for *D. lepeophtherii.* Numbers are shown in Table 5.

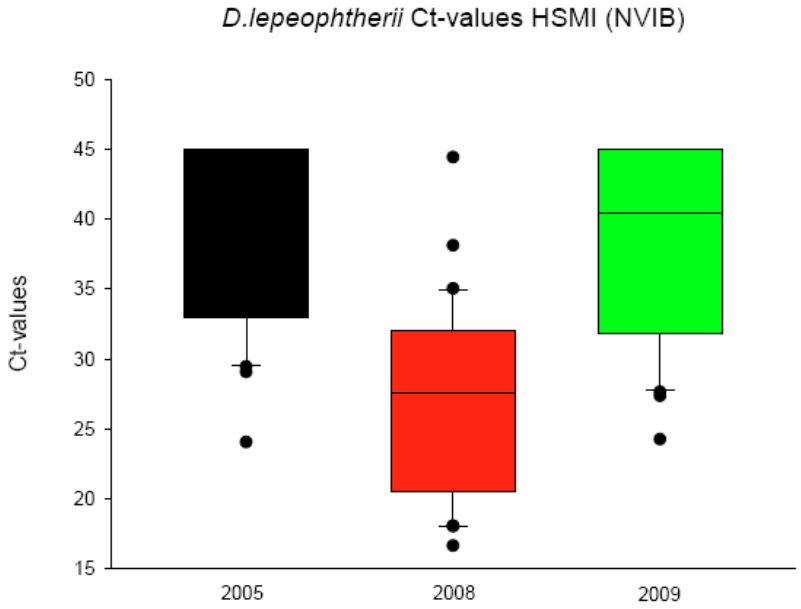


Figure 8- Real time RT-PCR results for *D. Lepeophtherii* specific analysis of HSMI diagnosed material from NVIB year 2005, 2008 and 2009. Results shown as Ct-value

Table 5 - Displays an overview of HSMI diagnosed cases and samples from the Norwegian Veterinary Institute Bergen (NVIB) investigated for *D. lepeophtherii.*

Year	Total cases	Individual samples	Rejected samples	Positive samples
2005	9	35	0	25
2008	8	30	0	30
2009	10	38	3	19

Cardiomyopathy syndrome

D. lepeophtherii Ct- values for 2005, 2008 and 2009 CMS samples (NVIB)

Average real time RT-PCR result for CMS Bergen 2005 was 36,7 ± 4,9, for 2008 34,4 ± 1,6 and for 2009 33,0 ± 2,3 (all values given as mean Ct-value ± standard deviation) (Fig. 9). Generally there are fewer cases and individuals diagnosed with CMS in the southern part of Norway compared to mid and Northern Norway. In 2005 26 samples representing 9 cases were examined. Five samples were rejected due to low RNA quality. In 2008 3 cases with 9 individual samples were examined. In 2009 6 diagnostic cases with 16 individuals were examined. Numbers are shown in Table 6.

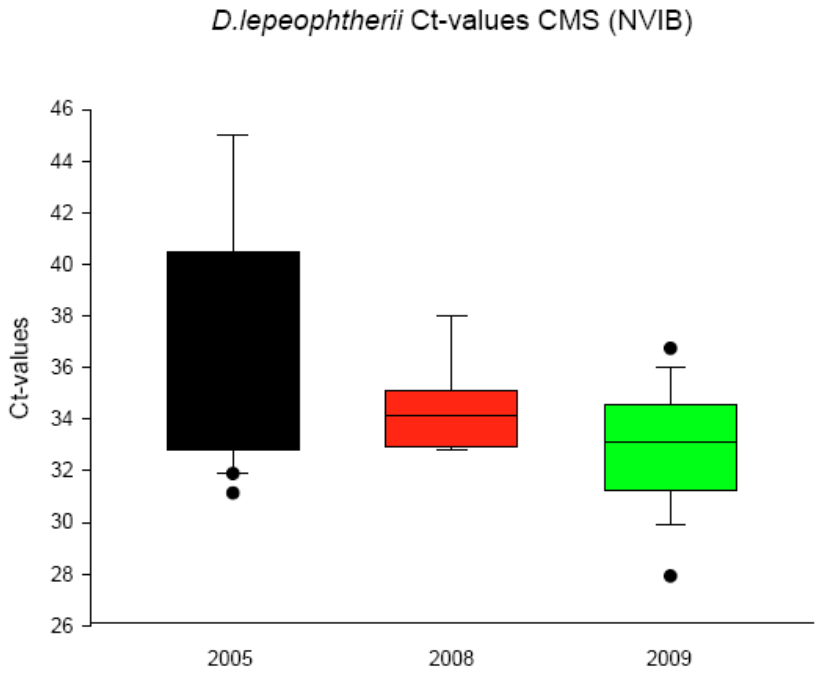


Figure 9- Real time RT-PCR results for *D. Lepeophtherii* specific analysis of CMS diagnosed material from NVIB year 2005, 2008 and 2009. Results shown as Ct-values

Table 6 - Displays an overview of CMS diagnosed cases and samples from the Norwegian Veterinary Institute Bergen (NVIB) investigated for *D. lepeophtherii.*

Year	Total cases	Individual samples	Rejected samples	Positive samples
2005	9	26	5	17
2008	3	9	0	9
2009	6	16	0	16

Proliferative gill inflammation

D. lepeophtherii Ct- values for 2005, 2008 and 2009 PGI samples (NVIB).

Average real time RT-PCR result for PGI Bergen for 2005 was 30,0 ± 3,0, for 2008 23,0 ± 4,0 and for 2009 29,3 ± 5,1 (all values are given as mean Ct-value ± standard deviation) (Fig.10). All samples studied were found positive for *D. lepeophtherii.* In 2005 23 individuals from 9 cases were examined. In 2008 27 individuals distributed on 7 cases were examined. In 2009 25 individuals distributed on 7 cases were examined. Two samples from 2005 and one sample from 2009 were rejected due to low RNA quality. Numbers are shown in Table 7.

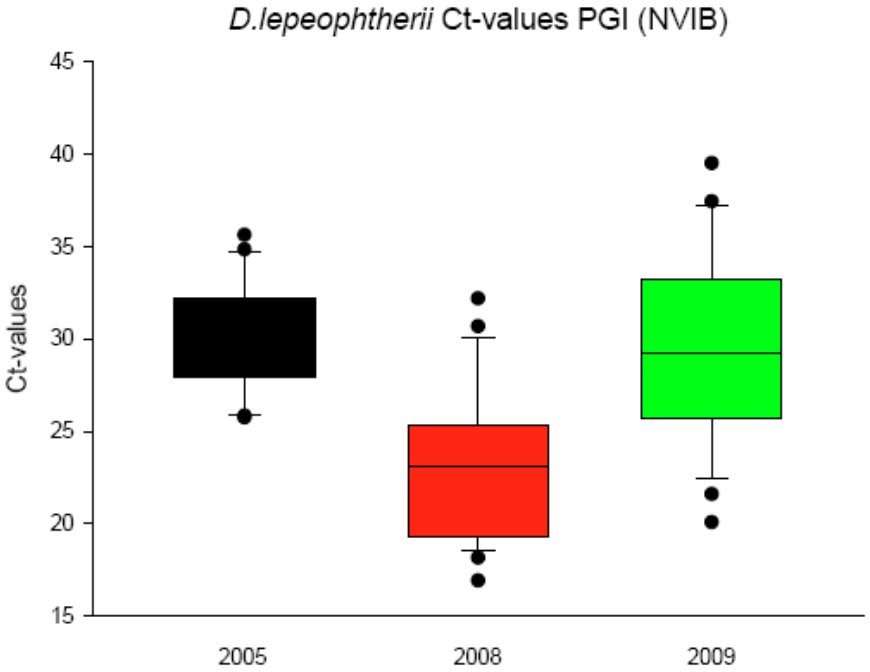


Figure 10 - Real time RT-PCR results for *D. Lepeophtherii* specific analysis of PGI diagnosed material from NVIB year 2005, 2008 and 2009. Results shown as Ct-values

Table 7 - Displays an overview of PGI diagnosed cases and samples from the Norwegian Veterinary Institute in Bergen (NVIB) investigated for *D. lepeophtherii*

Year	Total cases	Individuals samples	Rejected samples	Positive samples
2005	9	23	2	21
2008	7	27	0	27
2009	7	25	1	24

Autumn disease

D. lepeophtherii Ct- values 2008 (NVIB)

Average realtime result for autumn disease Bergen 2008 were 22,1 ± 5,0 (value is given as mean Ct-value ± standard deviation). Twenty-eight individuals distributed on 7 cases were examined (Fig. 11). All samples tested positive for *D. lepeophtherii*.

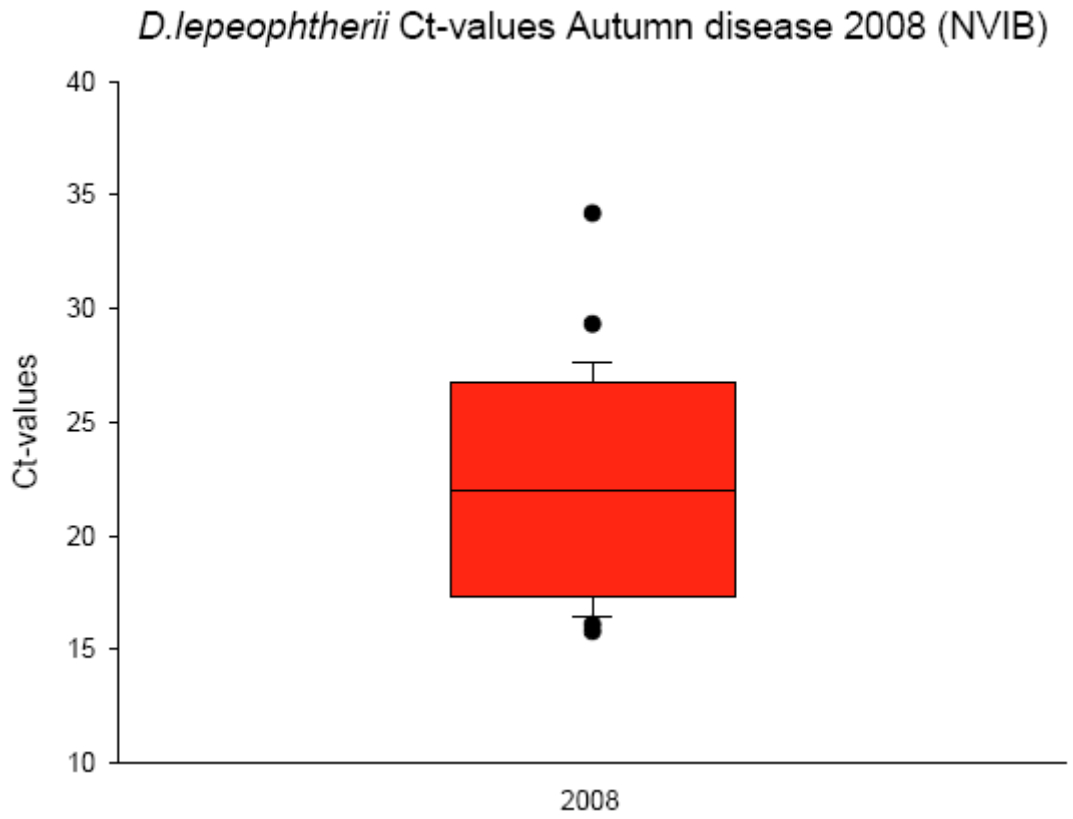


Figure 11 - Real time RT-PCR results for *D. Lepeophtherii* specific real time PCR on autumn disease diagnosed material from NVIB year 2008. Results shown as Ct-values.

Table 8 -Displays an overview of autumn disease diagnosed cases and samples from NVIB investigated for *D. lepeophtherii*.

Year	Total cases	Individual samples	Rejected samples	Positive samples
2008	7	28	0	28

Real time RT-PCR results from material obtained from NVIH

Desmozoon lepeophtherii Ct- values from 2009 PD, HSMI, CMS and PGI samples NVIH

Since there was only one outbreak of pancreas disease in Northern Norway in 2009, only this case was examined. Five out of 16 fish were positive for *D. lepeophtherii* in the real time RT-PCR analysis. Average real time result for PD Harstad 2009 was 43,4 ± 2,61 (all values given as mean Ct-value ± standard deviation). Heart and skeletal muscle inflammation samples examined had 42 individuals distributed on 11 cases. Real time RT-PCR result for HSMI NVIH 2009 was 38,2 ± 5,2. Results from samples with CMS from NVIH were based on 10 cases with 27 samples. One sample was rejected due to low quality RNA. Average real time result for CMS NVIH 2009 was 41,8 ± 4,9. From 6 cases with 16 fish alltogether suffering from PGI, only one individual proved to be positive for the parasite. The realtime results from PGI 2009 Harstad was 44, 9 ± 0,5. Ct-values shown in Fig. 12. Numbers of cases and individuals are shown in Table 9.

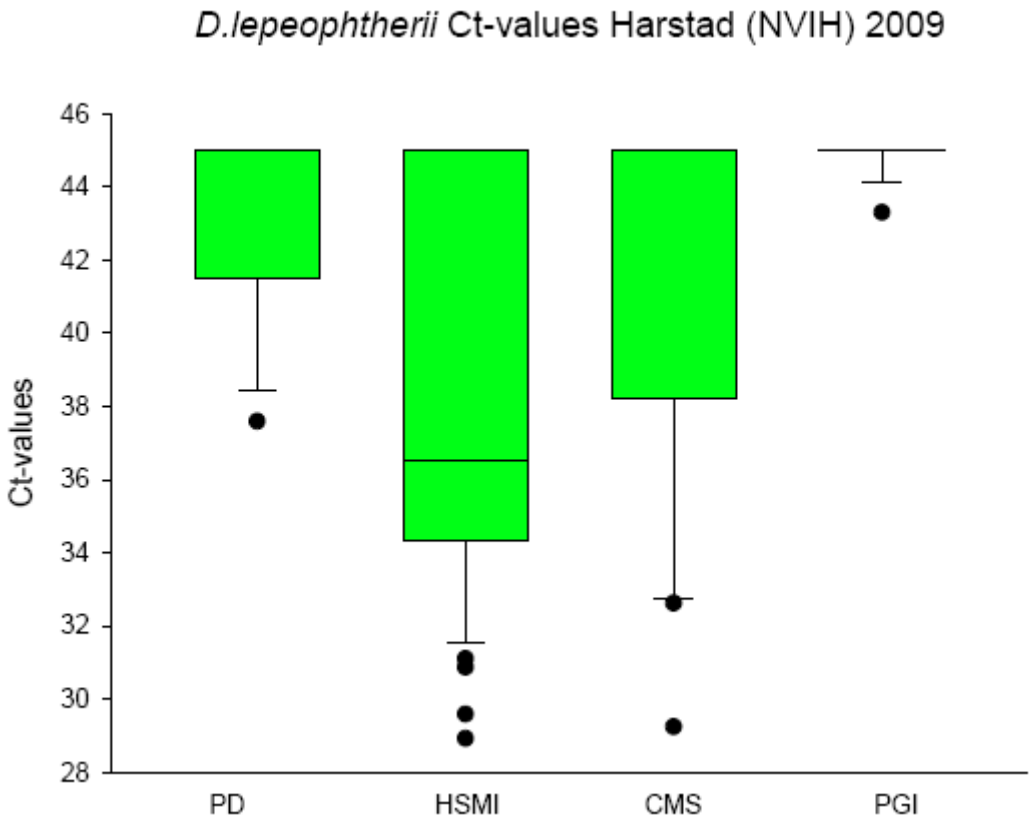


Figure 12 - Real time RT-PCR results for *D. Lepeophtherii* specific analysis of PD, HSMI, CMS and PGI diagnosed material from NVIH year 2009. Results shown as Ct-values.

Table 9 - Displays an overview of cases and samples from the Norwegian Veterinary Institute Harstad (NVIH) investigated for *D. lepeophtherii.*

Diagnosis	Total cases	Individual samples	Rejected samples	Positive samples
PD	1	16	0	5
HSMI	11	42	0	29
CMS	10	27	1	9
PGI	6	14	0	1

Calcofluor white stain

Results from the calcofluor white staining showed that *D. lepeophtherii* was present in 4 out of the 11 samples investigated (Fig. 13). Positive results indicating the microsporidium are shown as blue lighted spots in the pictures. The sample which represented PD 2008 (Fig. 13a) did not have any signs of the parasite. From the samples with HSMI 2008 (Fig.13b and c), one tested positive. HSMI samples from the year 2009 (Fig. 13d and e) were also negative. The CMS sample (Fig. 13f) was negative. The 2008 PGI sample tested negative (Fig. 13g) but the two 2009 PGI samples tested positive (Fig. 13h and i). Both 2008 autumn disease samples tested positive (Fig. 13j and k). Particularly the sample representing the individual with lowest Ct-value in the whole project (Fig. 13k) had a massive infiltration of the parasite in the kidney tissue.

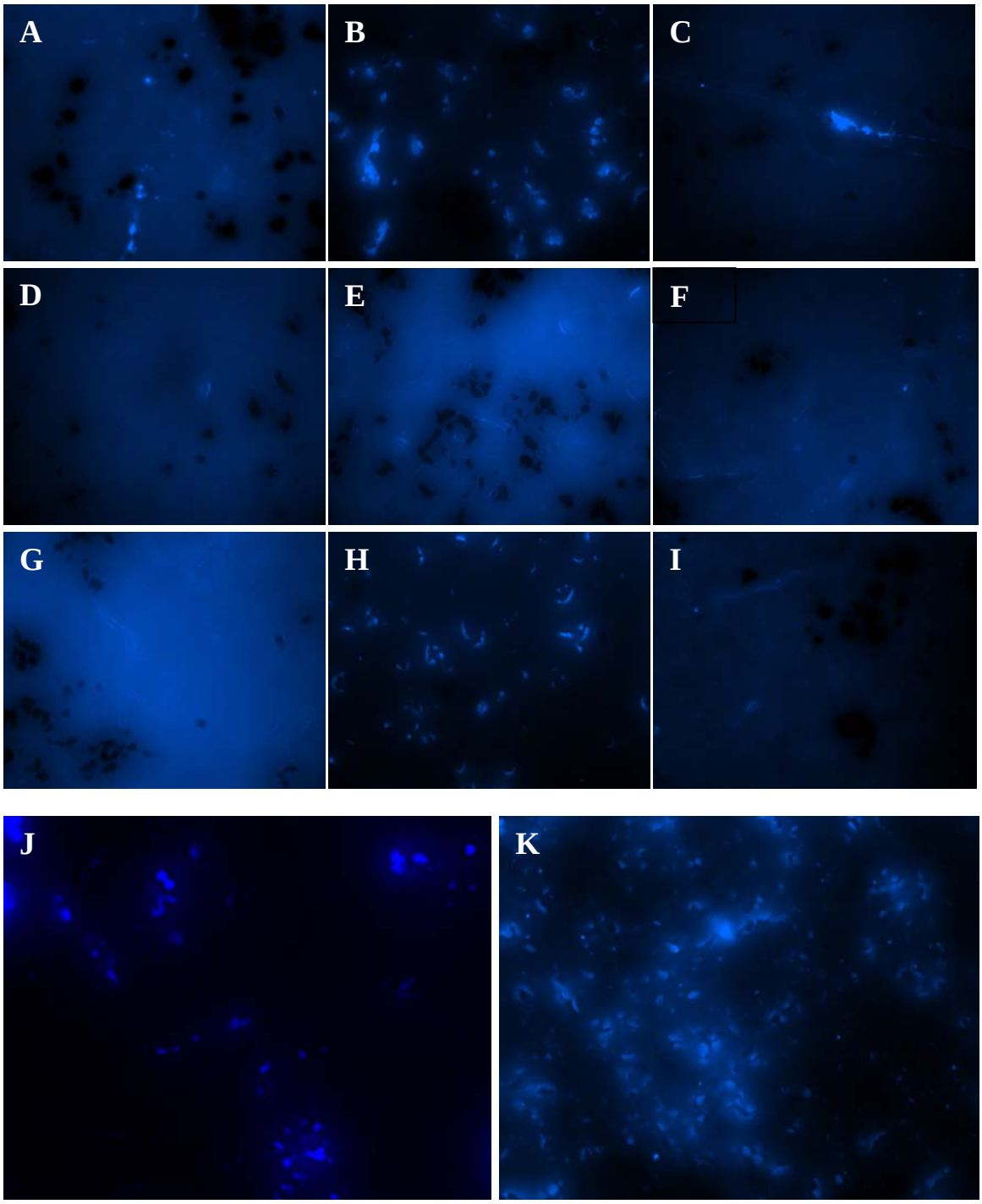


Figure 13 - Calcofluor white stain material with individuals representing average Ct-value for each represented year and disease. PD 2008 (A), HSMI 2008, (B and C), HSMI 2009 (D and E), CMS 2008 (F), PGI 2008 (G), PGI 2009 (H and I), autumn disease (J and K).

Discussion

In this work we have used the name *Desmozoon lepeophtherii* for the microsporidian suggested associated with the "autumn disease". Choice of name is according to the official name policy of the Norwegian Veterinary Institute (NVI).

Material and methods

The primary goal for this study was to examine historical diagnostic material from the years 2005, 2008 and 2009 for the presence of the microsporidian parasite *D. lepeophtherii*. Fish individuals that have been diagnosed with PD, HSMI, CMS and PGI were tested along with a "new" condition, called autumn disease from 2008 which were presumed associated with *D. lepeophtherii*. As a secondary goal we wanted to establish the use of blocks with formalin fixed paraffin embedded (FFPE) material in real time PCR based studies.

From the Veterinary Institute in Bergen, all samples used in the study were single organ (renal tissue) paraffin blocks. The material from the Veterinary Institute in Harstad was multiorgan blocks. Based on experience from the RNA purification of the FFPE materials, it was easier to perform optimal RNA purification from single organ material compared to the multiorgan material due to problems with estimating the correct amount of tissue to be used in the FFPE assay.

The first samples purified were performed according to the user manual from 2006 (RNeasy®FFPE handbook QIAGEN). In September 2010 QIAGEN did some changes to the kit, and these kits were used further in the project except once, when a delivery failure led to an old kit being delivered. When purifying with this "old" kit, the RNA yield was much lower than expected. This was probably because of storage at too high temperatures by the supplier. Formalin fixed and paraffin embedded tissues are known to have more fragmented RNA quality than RNA obtained directly from fresh tissues and tissues stored in RNAlater™. The RNeasy®FFPE kit used in this project is specialized to preserve and optimize recovering of fragmented RNA from the FFPE. Samples that pointed out to have low RNA yield, was purified twice to eliminate possible human errors. If they still had a low yield after the second purification, these samples were rejected. RNA quality and yield are dependent on the quality

of the material. If the material is based on dead fish with cadaverous changes this will reduce the RNA quality. Insufficient fixation of the material will also lead to increased RNA detoriation [66]. To take care of the organ quality, the tissue has to be handled correctly. According to the NVI's user guide for sampling fish, the criteria for good fixation are 1:10 ratio of tissue and formalin and 5 mm maximum thickness of the tissue (http://www.campec.net/brukerhandboka/Fisk/F001_fisk_ram.htm). Another issue that can be related to low RNA yield is errors regarding embedding of the organs. When preparing the paraffin embedded blocks, oxygen may come inside which then induce degeneration of the RNA. During the work with sectioning the FFPE blocks we experienced that some of the block had cavities and thereby exposing the tissue to oxygen. This would again, according to the RNeasy®FFPE user manual, lead to a decrease in RNA yield. All samples originally purified and those used in the project are listed in the appendix (Appendix 2). Of 498 samples used fore RNA purification 30 samples were rejected after purification. This indicates that most FFPE material has intact RNA. After purification, the total eluted RNA samples were stored at - 80ºC for further use for RT-PCR.

PCR

For the detection of the microsporidian parasite, real time RT-PCR was used with primers and probe specific for *D. lepeophtherii*. All handling of the purified RNA was minimized and during handling time the material was kept on ice. Purified RNA may have variable quality, and may also be unstable if stored over a period of time [66]. As mentioned above the housekeeping gene was used in the study to check the quality of the purified RNA material. The real time RT-PCR results of the EF1A amplification indicated a good quality of the purified material. Of 449 samples analyzed with real time RT-PCR there was no difference in the average Ct-value of material from 2005, 2008 and 2009 (see Appendix 2). This indicates that material older than 2005 also probably will be of sufficient quality for this type of analysis. Of 468 samples analyzed with real time RT-PCR 449 samples were rejected due to insufficient EF1A amplification. This indicates that 96 % of the samples had good RNA quality.

Autumn disease

The autumn disease was as mentioned above, first described in 2008. *D. lepeohtherii* was later suggested associated with the condition [50]. The results from the real time PCR amplifications show large amounts of *D. lepeophtherii* in kidney tissue from fish suffering from autumn disease. It is clearly that the fish individuals suffer from a systemic infection of the parasite. All autumn disease samples are positive for *D. lepeohtherii* with an average Ct-value of 22,1. The PCR results therefore seem to confirm that *D. lepeophtherii* is associated with autumn disease. The lowest Ct-value registered was 15,8, indicating that fish with autumn disease related clinical changes have large amount of spores. Comparison of real time RT-PCR results with histopathology based diagnostic answers showed that fish with clinical changes associated with autumn disease, seemed to have Ct-values below 25. Ct-values from 25-28 represented an intermediate area with unclear clinical association. In fish with Ct-values from 29 and up there were no clinical association.

Proliferative gill inflammation

All samples with the diagnosis PGI (years 2005, 2008 and 2009) at NVIB were found positive for *D. lepeophtherii* (average Ct-values were 30,0 ± 3,0 for 2005, 23,0 ± 4,0 for 2008 and 29,3 ± 5,1 for 2009 respectively). The mean Ct-values found in the PGI material were o lower than what was observed in fish diagnosed with PD, HSMB and CMS. There is a significant increase in the amount of *D. lepeophtherii* in fish from 2008 compared to both 2005 and 2009 ($p<0,001$). This is the same year as the autumn disease was reported to occur. Twenty out of 27 investigated samples show Ct-values indicating high amounts of *D. lepeophtherii* in tissue material this year (Ct-values below 25). The mean Ct-value for 2008 is almost the same as was found for the autumn disease. This could indicate that *D. lepeophtherii* might be connected to the diagnosis of PGI in 2008. Ct-values from 2005 and 2009 are apparently the same with mean values around 30. Among these samples only a few individuals have Ct-values which could indicate that the fish might have clinical problems with the parasitic infection. In the other individuals the parasite is present, but the Ct-values are regardless too high to indicate clinical illness. In the NVIH material from Northern Norway, 6 cases were studied. Only one individual had positive PCR results. However the Ct-value was so high, that we can only suggest the presence of the parasite. *D. lepeophtherii* is therefore not

associated with PGI in the material from Northern Norway. PGI is a condition with an unclear etiology and with probably several possible causal agents. This is also reflected in the results here. Based on these results, we can not exclude that there is a relationship between *D. lepeophtherii* and the diagnosis PGI. It looks like the parasite may be one of several factors that contribute to PGI. Higher *D. lepeophtherii* burden in PGI diagnosed salmon has also lately been confirmed by others [55 69]. It is, however, unclear if the microsporidium is a primary causal agent or just a secondary invador of the diseased gills.

.

Pancreas disease

The PCR results from cases with PD in Southern Norway (2005, 2008 and 2009 NVIB) show low presence of *D. lepeophtherii* (mean Ct-values for 2005 41,3 ± 5,2, for 2008 36,0 ± 7,1 and for 2009 38,7 ± 5,8). In the NVIH material there was just one case from 2009. Mean Ct-value for the samples in this case was 43,4 ± 2,6. None of the individuals had results indicating any clinical implication of the infection. Around 50% of the investigated individuals in the NVIB material were positive for the microsporidium. Around 30% of the individuals in the NVIH material were positive. In the NVIB material there was tendency for higher amounts of the microsporidium in the 2008 material. This is corresponding to what was seen in the PGI material and support the impression of a higher total amount of *D. lepeophtherii* in farmed salmon in 2008. Altogether there is no support in the results for any association between PD and *D. lepeophtherii* as suggested by Nylund *et al*., [51]. This is also supported by the fact that the numerical top for PD diagnosis is during the summer months while the top for the autumn disease cases is as suggested by the name, during the autumn months.

Heart and skeletal muscle inflammation

Mean PCR Ct-values for the HSMI material from NVIB were for 2005 37,4 ± 6,2, for 2008 27,2 ± 6,9 and for 2009 38,4 ± 7,1. For 2005 and 2008 the values are approximately the same as found in the PD material. The mean Ct-values from both 2005 and 2009 are high and around the cut off value for the assay. Most of the samples from these years are positive for *D. lepeophtherii*, but in such low amounts that no clinical implication is expected. All samples

examined from year 2008 were positive for *D. lepeophtherii*. Several of the individuals have microsporidium burden in the same amount as have been seen associated with clinical changes. As seen from the official numbers of HSMI cases at NVIB (Fig. 14), 2008 was a year with few HSMI diagnosis. The PCR therefore seems to reflect the general higher *D. lepeophtherii* burden in farmed salmon this year, rather than a clinical association between *D. lepeophtherii* and HSMI. Most of the 2008 NVIB cases studied were also sampled during the autumn months resulting in a general higher *D. lepeophtherii* pressure. The HSMI (NVIH) material from Northern Norway showed mean Ct-results around the cut off value (38,2 ± 5,2). When one then takes into consideration that there are more cases with HSMI in Northern Norway than in Southern Norway (see Fig. 15) it appears to be no association between *D. lepeophtherii* and HSMI.

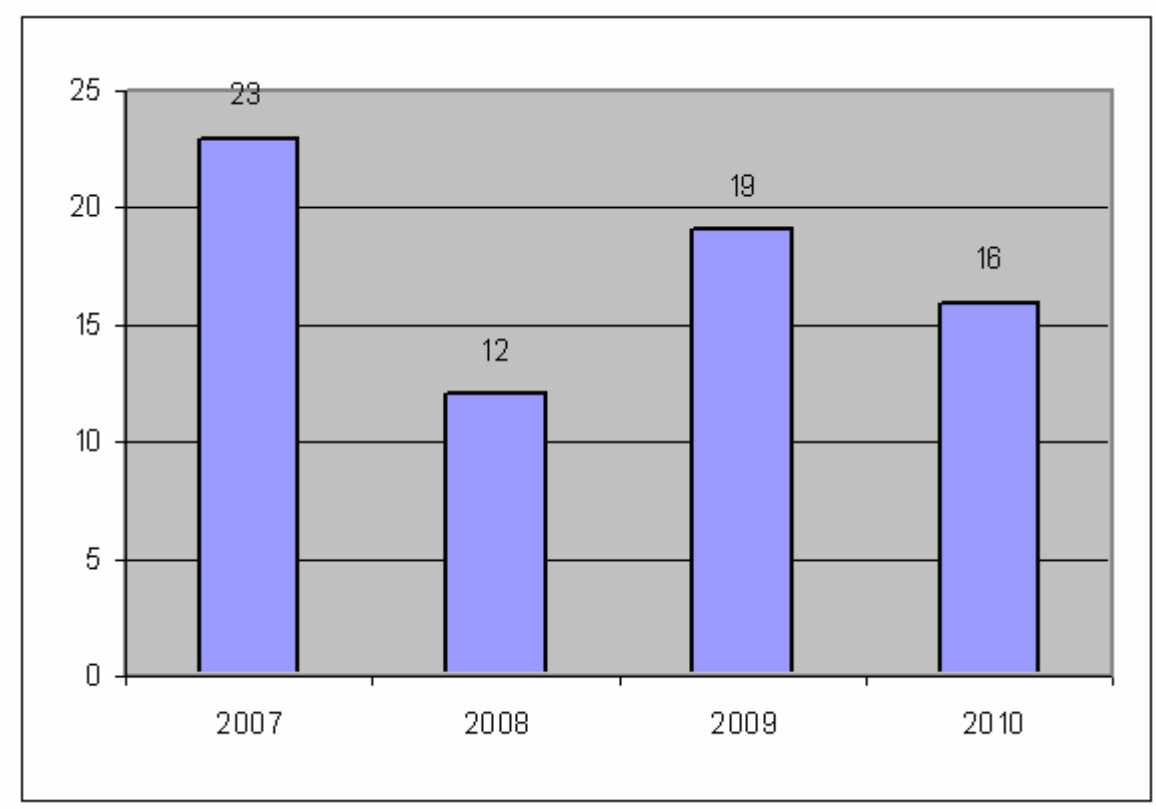


Figure 14 - Number of cases diagnosed with HSMI at NVIB from 2007 to 2010. The figures show a drop in the number of cases in 2008. (Official numbers presented by NVIB at the 2011 user meeting)

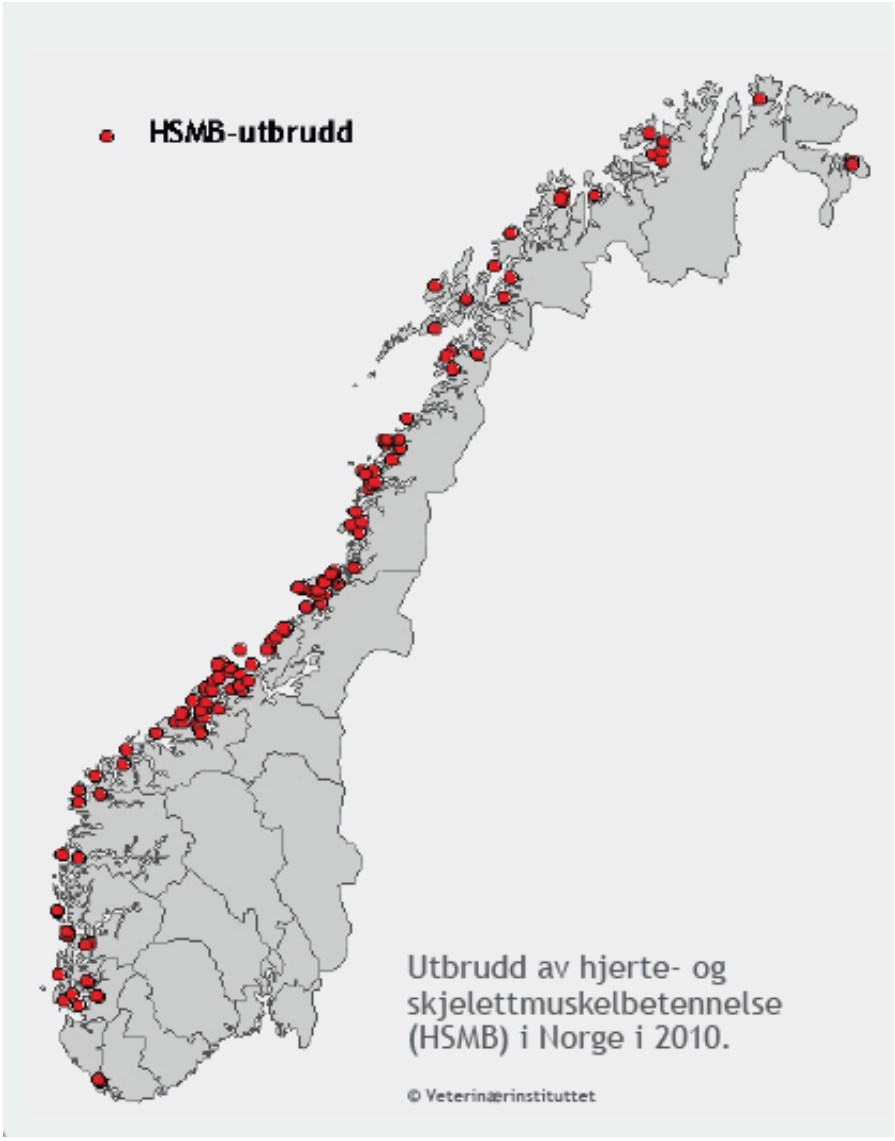


Figure 15 - Map showing location of cases diagnosed with HSMI in 2010. (Map from the fish health report from the NVI) [7].

Cardiomyopathy syndrome

PCR results for CMS material from NVIB were for 2005 were 36,7 ± 4,9, for 2008 34,4 ± 1,6 and for 2009 33,0 ± 2,3. There are few CMS cases each year at NVIB. The cases from the 3 years studied in this project, covered just 46 individuals. The results are therefore suboptimal for mapping any potential connection between *D. lepeophtherii* and CMS. However, mean Ct-values are so high that no clinical implication from the infection can be expected. The results from NVIH showed even lower values for the CMS material (41,8 ± 4,9) although one third of the samples were positive for *D. lepeophtherii*. Altogether these results indicate lack of association between *D. lepeophtherii* and CMS.

Calcofluor white stain

The microsporidian sporewall contains chitin. The nonspecific chemofluorescent calcofluor binds to material such as cellulose and chitin. Calcofluor white stain was therefore used to detect *D. lepeophtherii* in the analyzed material. Since 1982 [74], this method have been used to detect microsporidian spores in scientific connections. Calcofluor has an optimal excitation at wavelength 347 nm (DAPI). This type of stain is, as mentioned, nonspecific, and fungal spores and cellulose containing material present in the sample could also fluorescence. This could affect the final results. Still the salmon tissue investigated in this project was from individuals from the seawater phase, and the probability of any other fungal organism present in the kidney samples was minimal. Also small particles from paper could interfere with the calcofluor white stain and give false positives. The fluorescence organisms or particles in tissue samples had oval to round form. This indicates that microsporidian spores were detected. The samples chosen to be tested for the calcofluor white stain were selected based on the mean Ct-value the represented year. Results from PD 2008 was as expected negative in terms of the given average Ct-value (33,5). Two samples with HSMI 2008, and two from 2009 were tested for the presence of the microsporidium to study the differences between these years. The two samples from 2009 were found negative, but one of the 2008 samples was positive and the other negative. From the mean Ct-value this year, the result was expected to be negative but one sample came up positive (Fig. 13b). The sample representing CMS 2008 was negative with no signs of the parasite. This was expected because of the high average Ct-value proven from the PCR amplification. From the picture (Fig. 13f), the fluorescing structures observed are related to section artifacts. Regarding PGI, one sample from 2008, and two from 2009 (Fig.13g, h, i) were examined. It was expected that the sample from 2008 should be positive due to the low Ct-value, but this slice did not have any sign of the parasite (Fig. 13g). Also for 2009 only one of the PGI samples was found positive. As expected, both samples with autumn disease were found positive reflecting a high amount of spores in these tissues. The reason for the negative samples diverging from the PCR results is uncertain. During the experiment it was observed that some of the sections appeared to be insufficient deparaffinized and rehydrated, when KOH and calcofluor were applicated. This might have influenced the result and made some of the preparations appear falsely negative. The autumn disease material shows large amount of *D. lepeophtherii* in renal tissue from the affected individuals. This supports the observed association between the microsporidian and the autumn disease. Appearance of *D. lepeophtherii* in the PGI material could, however, not

be confirmed with this method. As mentioned above, this could be due to methodological problems.

Summary

The results show no association between *D. lepeophtherii* and the diagnoses PD, HSMI and CMS in the examined material. In general, the real time PCR results show a tendency for more *D. lepeophtherii* material in NVIB derived PD, HSMI and PGI samples from 2008 compared to 2005 and 2009. For the PGI material the results suggest a possible association. This association is especially pronounced in the NVIB material from 2008. For the autumn disease material and the PGI 2008 material the results show an association to *D. lepeophtherii.* It is however unclear if the *D. lepeophtherii* is a primary disease cause or a secondary effect of the disease. Comparison between the NVIB and NVIH material show a higher *D. lepeophtherii* burden in the material from Southern Norway. In the NVIH material only minimal amounts of the parasite is detected. This may be due to the biology of the microsporidium. Microsporidian development appears to be temperature dependent. Other studies have concluded that microsporidian parasites (*Loma salmonae* [3,4,64], *Enterocytozoon Salmonis* [2] and *Glugea Stephani* [57]) develop faster with higher temperatures. For optimal growth and germination of *D. lepeophtherii*, the temperature has to be about 15ºC. This is to reach optimal microsporidian replication conditions [53,55]. Experience from the salmon louse laboratory at the Institute for Marine Research also supports this theory (Prof. Frank Nilsen personal comm.). There is also a lower louse burden in Northern Norway compared to the southern part of the country. These two factors can explain the lower presence of *D. lepeophtherii* in Northern Norway.

Conclusive remarks and future perspectives

- The results from this study indicate lack of association between *D. lepeophtherii* and the diseases PD, HSMI and CMS. There is, however, a possible link between *D. lepeophtherii* and the diseases PGI and, autumn disease. This association is most pronounced for the diagnostic material from 2008.
- The low *D. lepeophtherii* burden in the material from Northern Norway enhances the results showing lack of association between *D. lepeophtherii* and PD, HSMI and CMS. There is no connection between occurrence of disease and *D. lepeophtherii* burden. It also shows occurrence of PGI without a microsporidian infection.
- The results show *D. lepeophtherii* infection to be common in farmed salmon in Southern Norway.
- Calcofluor white stain can be used for the detection of microsporidian presence in tissue samples.
- Use of formalin fixed paraffin embedded material is suitable for this kind of study.

The results from this study open for future studies on archived formalin fixed paraffin embedded material.

Reference List

1. **Amin A. B. and J**.**Trasti** 1988. Endomyocarditis in Atlantic salmon in Norwegian seafarms. Bulletin for the Europeian Association of Fish Pathologists **8**:70-71.

2. **Antonio, D. B. and R. P. Hedrick**. 1995. Effect of Water Temperature on Infections with the Microsporidian *Enterocytozoon salmonis* in Chinook Salmon. Diseases of Aquatic Organisms **22**:233-236.

3. **Beaman, H. J., D. J. Speare, and M. Brimacombe**. 1999. Regulatory effects of water temperature on *Loma salmonae* (Microspora) development in rainbow trout. Journal of Aquatic Animal Health **11**:237-245.

4. **Becker, J. A., D. J. Speare, and I. R. Dohoo**. 2006. Interaction of water temperature and challenge model on xenoma development rates for *Loma salmonae* (Microspora) in rainbow trout, *Oncorhynchus mykiss* (Walbaum). Journal of Fish Diseases **29**:139-145.

5. **Bigliardi, E.** 2001. Microsporidia, enigmatic parasites. Italian Journal of Zoology **68**:263-271.

6. **Bigliardi, E. and L. Sacchi**. 2001. Cell biology and invasion of the microsporidia. Microbes and Infection **3**:373-379.

7. **Bornø, G., S. Sviland, B. B. Jensen, A. Tarpai, A. H. Garseth, H. R. Skjelstad, , R. Johansen, O. B. Dale, C. Fritsvold, H. Nilsen, Ø. Vågnes, K. Flesja, S.Aure, D. Colquhoun, I. Ørpetveit, H. Hansen, P. A. Heuch, and B. Hjeltnes.** 2010. Helsesituasjonen hos laksefisk 2010. National Veterinary institute, Norway. 1-24.

8. **Brocklebank, J. and S. Raverty**. 2002. Sudden mortality caused by cardiac deformities following seining of preharvest farmed Atlantic salmon (*Salmo salar*) and by cardiomyopathy of postintraperitoneally vaccinated Atlantic salmon parr in British Columbia. Canadian Veterinary Journal-Revue Veterinaire Canadienne **43**:129-130.

9. **Brun, E., T. Poppe, A. Skrudland, and J. Jarp**. 2003. Cardiomyopathy syndrome in farmed Atlantic salmon *Salmo salar*: occurrence and direct financial losses for Norwegian aquaculture. Diseases of Aquatic Organisms **56**:241-247.

10. **Bruno, D. W. and P. A. Noguera**. 2009. Comparative experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms **87**:235-242.

11. **Cali, A. and P. M. Takvorian**. 1999. Developmental morphology and life cycles of the microsporidia. *In*: Wittner, M. and L. M. Weiss (eds.). The microsporidia and Microsporidiosis. American Society for Microbiology, Washington.D.C.85-128.

12. **Curgy, J. J., J. Vavra, and C. Vivares**. 1980. Presence of Ribosomal-Rnas with Prokaryotic Properties in Microsporidia, Eukaryotic Organisms. Biologie Cellulaire **38**:49-51.

13. **Degraaf, D. C., H. Raes, and F. J. Jacobs**. 1994. Spore Dimorphism in *Nosema Apis* (Microsporida, Nosematidae) Developmental Cycle. Journal of Invertebrate Pathology **63**:92-94.

14. **Dunn, A. M. and J. E. Smith**. 2001. Microsporidian life cycles and diversity: the relationship between virulence and transmission. Microbes and Infection **3**:381-388.

15. **Erickson, B. W. and R. S. Blanquet**. 1969. Occurrence of Chitin in Spore Wall of *Glugea Weissenbergi*. Journal of Invertebrate Pathology **14**:358-364.

16. **Ferguson, H. W., R. T. Kongtorp, T. Taksdal, D. Graham, and K. Falk**. 2005. An outbreak of disease resembling heart and skeletal muscle inflammation in Scottish farmed salmon, *Salmo salar* L., with observations on myocardial regeneration. Journal of Fish Diseases **28**:119-123.

17. **Ferguson, H. W., T. Poppe, and D. J. Speare**. 1990. Cardiomyopathy in Farmed Norwegian Salmon. Diseases of Aquatic Organisms **8**:225-231.

18. **Ferguson, H. W., R. J. Roberts, R. H. Richards, R. O. Collins, and D. A. Rice**. 1986. Severe Degenerative Cardiomyopathy Associated with Pancreas Disease in Atlantic Salmon, *Salmo Salar* L. Journal of Fish Diseases **9**:95-98.

19. **Freeman, M. A.** 2002. Potential biological control agents for the salmon louse *Lepeophtheirus salmonis* (Krøyer 1837). University of Stirling. PhD. 1-243.

20. **Freeman, M. A., A. S. Bell, and C. Sommerville**. 2003. A hyperparasitic microsporidian infecting the salmon louse, *Lepeophtheirus salmonis*: an rDNA-based molecular phylogenetic study. Journal of Fish Diseases **26**:667-676.

21. **Freeman, M. A. and C. Sommerville**. 2009. *Desmozoon lepeophtherii* n. gen., n. sp., (Microsporidia: Enterocytozoonidae) infecting the salmon louse *Lepeophtheirus salmonis* (Copepoda: Caligidae). Parasites & Vectors **2**.

22. **Fringuelli, E., H. M. Rowley, J. C. Wilson, R. Hunter, H. Rodger, and D. A. Graham**. 2008. Phylogenetic analyses and molecular epidemiology of European salmonid alphaviruses (SAV) based on partial E2 and nsP3 gene nucleotide sequences. Journal of Fish Diseases **31**:811-823.

23. **Fritsvold, C., R. Kongtorp, T. Taksdal, I. Orpetveit, M. Heum, and T. Poppe**. 2009. Experimental transmission of cardiomyopathy syndrome (CMS) in Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms **87**:225-234.

24. **Frixione, E., L. Ruiz, J. Cerbon, and A. H. Undeen**. 1997. Germination of Nosema algerae (Microspora) spores: Conditional inhibition by D2O, ethanol and Hg2+ suggests dependence of water influx upon membrane hydration and specific transmembrane pathways. Journal of Eukaryotic Microbiology **44**:109-116.

25. **Frixione, E., L. Ruiz, M. Santillan, L. V. Devargas, J. M. Tejero, and A. H. Undeen**. 1992. Dynamics of Polar Filament Discharge and Sporoplasm Expulsion by Microsporidian Spores. Cell Motility and the Cytoskeleton **22**:38-50.

26. **Germot, A., H. Philippe, and H. LeGuyader**. 1997. Evidence for loss of mitochondria in Microsporidia from a mitochondrial-type HSP70 in *Nosema locustae*. Molecular and Biochemical Parasitology **87**:159-168.

27. **Harrington B.J. and Hageage G.J**. 1991. Calcofluor white: tips for improving its use. Clinical Microbiological Newsletters **13**:3-5.

28. **Hodneland, K., A. Bratland, K. E. Christie, C. Endresen, and A. Nylund**. 2005. New subtype of salmonid alphavirus (SAV), Togaviridae, from Atlantic salmon *Salmo salar* and rainbow trout *Oncorhynchus mykiss* in Norway (vol 66, pg 113, 2005). Diseases of Aquatic Organisms **67**:181.

29. **Ishihara, R. and Y. Hayashi**. 1968. Some Properties of Ribosomes from Sporoplasm of *Nosema Bombycis*. Journal of Invertebrate Pathology **11**:377-385.

30. **Karlsbakk, E., A. Nylund, and F. Nilsen**. 2009. Protister.*In*: Kompendium i fiskesykdommer fiskeparasitter Bergen. 35-118.

31. **Keeling, P. J. and C. H. Slamovits**. 2004. Simplicity and complexity of microsporidian genomes. Eukaryotic Cell **3**:1363-1369.

32. **Keohane, E. M. and L. M. Weiss**. 1999. The structure, function and composition of the microsporidian polar tube.*In*: Wittner, M. and L. M. Weiss (eds.), The Microsporidia and Microsporidiosis. American Society for Microbiology, Washington D.C.196-224.

33. **Kongtorp, R. T., M. Halse, T. Taksdal, and K. Falk**. 2006. Longitudinal study of a natural outbreak of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases **29**:233-244.

34. **Kongtorp, R. T., A. Kjerstad, T. Taksdal, A. Guttvik, and K. Falk**. 2004. Heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L.: a new infectious disease. Journal of Fish Diseases **27**:351-358.

35. **Kongtorp, R. T. and T. Taksdal**. 2009. Studies with experimental transmission of heart and skeletal muscle inflammation in Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases **32**:253-262.

36. **Kongtorp, R. T., T. Taksdal, and A. Lyngoy**. 2004. Pathology of heart and skeletal muscle inflammation (HSMI) in farmed Atlantic salmon *Salmo salar*. Diseases of Aquatic Organisms **59**:217-224.

37. **Kvellestad, A., B. H. Dannevig, and K. Falk**. 2003. Isolation and partial characterization of a novel paramyxovirus from the gills of diseased seawater-reared Atlantic salmon (*Salmo salar* L.). Journal of General Virology **84**:2179-2189.

38. **Kvellestad, A., K. Falk, S. M. R. Nygaard, K. Flesja, and J. A. Holm**. 2005. Atlantic salmon paramyxovirus (ASPV) infection contributes to proliferative gill inflammation (PGI) in seawater-reared *Salmo salar*. Diseases of Aquatic Organisms **67**:47-54.

39. **Larsson, R.** 1986. Ultrastructure, function, and classification of microsporidia. Progress in Protistology **1**:325-390.

40. **Lom, J.** 2002. A catalogue of described genera and species of microsporidians parasitic in fish. Systematic Parasitology **53**:81-99.

41. **Lom, J. and F. Nilsen**. 2003. Fish microsporidia: fine structural diversity and phylogeny. International Journal for Parasitology **33**:107-127.

42. **Lovoll, M., J. Wiik-Nielsen, S. Grove, C. R. Wiik-Nielsen, A. B. Kristoffersen, R. Faller, T. Poppe, J. Jung, C. S. Pedamallu, A. J. Nederbragt, M. Meyerson, E. Rimstad, and T. Tengs**. 2010. A novel totivirus and piscine reovirus (PRV) in Atlantic salmon (*Salmo salar*) with cardiomyopathy syndrome (CMS). Virology Journal **7**.

43. **Matthews, R. A. and B. F. Matthews**. 1980. Cell and Tissue-Reactions of Turbot *Scophthalmus Maximus* (L) to *Tetramicra Brevifilum* gen. n., sp. n. (Microspora). Journal of Fish Diseases **3**:495-515.

44. **McLoughlin, M. F. and D. A. Graham**. 2007. Alphavirus infections in salmonids - a review. Journal of Fish Diseases **30**:511-531.

45. **McLoughlin, M. F., R. N. Nelson, J. I. McCormick, H. M. Rowley, and D. B. Bryson**. 2002. Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, *Salmo salar* L. Journal of Fish Diseases **25**:33-43.

46. **Munro, A. L. S., A. E. Ellis, A. H. Mcvicar, H. A. Mclay, and E. A. Needham**. 1984. An Exocrine Pancreas Disease of Farmed Atlantic Salmon in Scotland. Helgolander Meeresuntersuchungen **37**:571-586.

47. **Murphy, T. M., H. D. Rodger, E. M. Drinan, F. Gannon, P. Kruse, and W. Korting**. 1992. The Sequential Pathology of Pancreas Disease in Atlantic Salmon Farms in Ireland. Journal of Fish Diseases **15**:401-408.

48. **Naegeli, C.** 1857. Uber die neue Krankheit der Seidenraupe und vervandte Organismen. Beilage zur Botanischen Zeitung **15**:760-761.

49. **Nelson, R. T., M. F. McLoughlin, H. M. Rowley, M. A. Platten, and J. I. McCormick**. 1995. Isolation of A Toga-Like Virus from Farmed Atlantic Salmon *Salmo salar* with Pancreas Disease. Diseases of Aquatic Organisms **22**:25-32.

50. **Nylund, A.** 2009. Ingen ny sjukdom. Intrafish Mediagroup. http://www.intrafish.no/norsk/nyheter/article239157.ece?mobile=&lots=nonews

51. **Nylund, A. and et al**. 2010. Primære og sekundære årsaker til sykdom. Intervet agenda 1.

52. **Nylund, A., A. M. Kvenseth, and E. Isdal**. 1998. A morphological study of the epitheliocystis agent in farmed Atlantic salmon. Journal of Aquatic Animal Health **10**:43-55.

53. **Nylund, A., K. Watanabe, S. Nylund, C. E. Arnesen, and E. Karlsbakk**. 2009. Lakselus. Biologisk vektor for lakseparasitt. Naturen **4**:217-222.

54. **Nylund, A., K. Watanabe, S. Nylund, M. Karlsen, P. A. Saether, C. E. Arnesen, and E. Karlsbakk**. 2008. Morphogenesis of salmonid gill poxvirus associated with proliferative gill disease in farmed Atlantic salmon (*Salmo salar*) in Norway. Archives of Virology **153**:1299-1309.

55. **Nylund, S., Andersen L, Sævareid I, Plarre .H, K. Watanabe, C. E. Arnesen, E. Karlsbakk, and A. Nylund**. 2011. Diseases of farmed Atlantic salmon *Salmo salar* associated with infections by the microsporidian *Paranucleospora theridion*. Diseases of Aquatic Organisms **94**:41-57.

56. **Nylund, S., A. Nylund, K. Watanabe, C. E. Arnesen, and E. Karlsbakk**. 2010. *Paranucleospora theridion* n. gen., n. sp (Microsporidia, Enterocytozoonidae) with a Life Cycle in the Salmon Louse (*Lepeophtheirus salmonis*, Copepoda) and Atlantic Salmon (*Salmo salar*). Journal of Eukaryotic Microbiology **57**:95-114.

57. **Olson, R. E.** 1981. Effects of Low Temperature on the Development of the Microsporidan *Glugea stephani* in English Sole. *Parophrys vetulus*. Journal of Wildlife Diseases **17**:559-562.

58. **Olsvik, P. A., K. K. Lie, A. E. O. Jordal, T. O. Nilsen, and I. Hordvik**. 2005. Evaluation of potential reference genes in real-time RT-PCR studies of Atlantic salmon. Bmc Molecular Biology **6**.

59. **Palacios, G., M. Lovoll, T. Tengs, M. Hornig, S. Hutchison, J. Hui, R. T. Kongtorp, N. Savji, A. V. Bussetti, A. Solovyov, A. B. Kristoffersen, C. Celone, C. Street, V. Trifonov, D. L. Hirschberg, R. Rabadan, M. Egholm, E. Rimstad, and W. I. Lipkin**. 2010. Heart and Skeletal Muscle Inflammation of Farmed Salmon Is Associated with Infection with a Novel Reovirus. Plos One **5**.

60. **Poppe, T.** 1989. Pancreas disease of Atlantic salmon (*Salmo salar* L). post-smolts infected with infectious pancreatic necrosis virus (IPNV). Bulletin of The European Association of Fish Pathologists **9**:83-85.

61. **Poppe, T. T. and S. L. Seierstad**. 2003. First description of cardiomyopathy syndrome (CMS)-related lesions in wild Atlantic salmon *Salmo salar* in Norway. Diseases of Aquatic Organisms **56**:87-88.

62. **Ralps, J. R. M. R. A.** 1986. Hepatic microsporidiosis of juvenile grey mullet, *Chelon labrosus* (Risso), due to Microgemma hepaticus gen.nov. sp.nov. Journal of Fish Diseases **9**:225-242.

63. **Rodger, H. and T. Turnbull**. 2000. Cardiomyopathy syndrome in farmed Scottish salmon. Veterinary Record **146**:500-501.

64. **Sanchez, J. G., D. J. Speare, and R. J. F. Markham**. 2000. Normal and aberrant tissue distribution of *Loma salmonae* (Microspora) within rainbow trout, *Oncorhynchus mykiss* (Walbaum), following experimental infection at water temperatures within and outside of the xenoma-expression temperature boundaries. Journal of Fish Diseases **23**:235-242.

65. **Schram, T. A.** 1993. Supplementary descriptions of the developmental stages of *Lepeophtheirus salmonis* (Krøyer, 1837) (Copepoda: Caligidae), p. 30-50. *In* G. A. Boxshall and D. Defaye (eds.), Pathogens of wild and farmed fish: sea lice. Ellis Horwood.

66. **Seear, P. J. and G. E. Sweeney**. 2008. Stability of RNA isolated from post-mortem tissues of Atlantic salmon (*Salmo salar* L.). Fish Physiology and Biochemistry **34**:19-24.

67. **Shaw, R. W. and M. L. Kent**. 1999. Fish microsporidia. *In*: Wittner, M and L. M. Weiss (eds.). The Microsporidia and Microsporidiosis. American Society for Microbiology, Washington D.C. 418-446.

68. **Sprague, V., J. J. Becnel, and E. I. Hazard**. 1992. Taxonomy of Phylum Microspora. Critical Reviews in Microbiology **18**:285-395.

69. **Steinum, T., A. Kvellestad, D. J. Colguhoun, M. Heum, S. Mohammad, R. N. Grontvedt, and K. Falk**. 2010. Microbial and pathological findings in farmed Atlantic salmon *Salmo salar* with proliferative gill inflammation. Diseases of Aquatic Organisms **91**:201-211.

70. **Taksdal, T., A. B. Olsen, I. Bjerkas, M. J. Hjortaas, B. H. Dannevig, D. A. Graham, and M. F. McLoughlin**. 2007. Pancreas disease in farmed Atlantic salmon, *Salmo salar* L., and rainbow trout, *Oncorhynchus mykiss* (Walbaum), in Norway. Journal of Fish Diseases **30**:545-558.

71. **Undeen, A. H.** 1990. A Proposed Mechanism for the Germination of Microsporidian (Protozoa, Microspora) Spores. Journal of Theoretical Biology **142**:223-235.

72. **Vågnes, Ø. and O. B. Dale**. 2009. Haustsjuke. Norwegian Veterinary Institute. http://www.vetinst.no/nor/Faktabank/Alle-faktaark/Haustsjuke

73. **Vavra J. and L. M. Weiss**. 1999. Structure of microsporidia. *In*: Wittner, M. and L. M. Weiss (eds.). The microsporidia and microsporidiosis. American Society for Microbiology, Washington D.C.7-74.

74. **Vavra, J. and J. Chalupsky**. 1982. Fluorescence Staining of Microsporidian Spores with the Brightener Calcofluor White-M2R. Journal of Protozoology **29**:503.

75. **Voigt, K. and P. M. Kirk**. 2011. Recent developments in the taxonomic affiliation and phylogenetic positioning of fungi: impact in applied microbiology and environmental biotechnology. Applied Microbiology and Biotechnology **90**:41-57.

76. **Vossbrinck, C. R. and C. R. Woese**. 1986. Eukaryotic Ribosomes That Lack A 5.8S Rna. Nature **320**:287-288.

77. **Watanabe, K., M. Karlsen, M. Devold, E. Isdal, A. Litlabo, and A. Nylund**. 2006. Virus-like particles associated with heart and skeletal muscle inflammation (HSMI). Diseases of Aquatic Organisms **70**:183-192.

78. **Weidner, E.** 1976. The Microsporidian Spore Invasion Tube. The Ultrastructure, Isolation, and Characterization of the Protein Comprising the Tube. Journal of Cell Biology **71**: 23-34.

Appendix

Appendix 1 - Real-time RT-PCR set up

Estimate of template amount

RNA concentration:

- < 75ng/µl: 12,5µl template added
- 75-100ng/µl: 10µl template added
- 100-200ng/µl: 5µl template added
- 200-400ng/ul: 2,5µl template added
- > 400ng/µl: calculate amount and add 750ng

Table 10 - Mastermix NUC: (buffer, dNTP and enzyme mix from OneStep kit QIAGEN)

Reagent	Starting concentration	Final concentration	1-reaction
RNasefree water	-	-	(12,5.RNA)
RT-PCR buffer	5×	1×	4
ROX reference dye	1 mM	30nM	0,3
dNTP	10mM(each)	400µM (each)	0,8
F	60µM	0,6µM	0,2
R	60µM	0,6µM	0,2
MGB	17,5µM	0,17µM	0,2
MgCl2	25mM	1,25mM	1
RNaseOut	40U/µl	4U	0,1
RT-PCR enzyme mix	-	-	0,8
Total			7,6

Table 11- Mastermix EF1A: (buffer, dNTP and enzyme mix from OneStep kit QIAGEN)

Reagent	Starting concentration	Final concentration	1-reaction
RNasefree water	-	-	(12,5.RNA)
RT-PCR buffer	5×	1×	4
ROX reference dye	1 mM	30nM	0,3
dNTP	10mM(each)	400µM (each)	0,8
F	90µM	0,9µM	0,2
R	90µM	0,9µM	0,2
MGB	20µM	0,2µM	0,2
MgCl2	25mM	1,25mM	1
RNaseOut	40U/µl	4U	0,1
RT-PCR enzyme mix	-	-	0,8
Total			7,6

Table 12 - RT-PCR Thermal Cycler performed in Stratagene Mx 3005P QPCR System

Temperature	Time
50°C	30 min
95°C	15 min
94°C	30 sec
55°C	45 sec
72°C	45 sec

Appendix 2 - Fish data and PCR results

Table 13 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PD NVIB 2005.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
March	F75/05	Kidney	226,4	2,03	Neg.	26,41
March	F75/05	Kidney	301,8	2,1	Neg.	24,25
March	F75/05	Kidney	366,1	1,92	Neg.	27,09
March	F75/05	Kidney	131,4	1,92	Neg.	29,55
April	F98/05	Kidney	165,7	2,07	Neg.	23,75
April	F98/05	Kidney	132,1	2,03	Neg.	23,17
April	F98/05	Kidney	362,7	2,07	Neg.	22,19
April	F98/05	Kidney	117,4	1,96	Neg.	26,88
April	F117/05	Kidney	1274,6	2,03	Neg.	23,28
April	F117/05	Kidney	502,1	2,04	Neg.	25,40
April	F117/05	Kidney	213,8	2,07	Neg.	25,70
April	F117/05	Kidney	301,6	2,06	Neg.	25,44
April	F122/05	Kidney	441,4	2,00	Neg.	24,34
April	F122/05	Kidney	124,4	2,05	Neg.	25,80
April	F122/05	Kidney	52,4	2,00	Neg.	27,56
April	F122/05	Kidney	640,7	2,07	Neg.	22,71
July	F228/05	Kidney	96,7	2,03	35,10	25,57
July	F228/05	Kidney	76,4	2,00	32,37	25,59
July	F228/05	Kidney	188,4	2,05	29,46	25,84
July	F228/05	Kidney	198,1	2,00	32,98	32,88
August	F240/05	Kidney	46,4	2,16	Neg.	25,27
August	F240/05	Kidney	339,9	1,99	Neg.	24,15
August	F240/05	Kidney	386,2	2,06	Neg.	23,00
August	F240/05	Kidney	516,3	2,00	Neg.	22,48
September	F287/05	Kidney	424,3	1,99	Neg.	28,37
September	F287/05	Kidney	518,8	2,01	43,19	22,88
September	F287/05	Kidney	856,7	2,03	Neg.	22,90
September	F295/05	Kidney	374,2	2,02	34,41	23,19
September	F295/05	Kidney	1037	1,99	34,22	23,32
September	F295/05	Kidney	336,2	2,03	Neg.	22,01
October	F312/05	Kidney	163,2	2,00	34,97	23,27
October	F312/05	Kidney	221,3	2,06	36,22	23,78
October	F312/05	Kidney	173,9	2,06	39,20	23,73
October	F312/05	Kidney	318,3	2,06	35,44	21,95
December	F375/05	Kidney	142,5	2,04	36,29	22,63
December	F375/05	Kidney	245,1	2,04	Neg.	25,97
December	F375/05	Kidney	245,5	2,07	Neg.	23,99
December	F375/05	Kidney	193,2	1,93	33,14	24,48

Table 14 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PD 2008 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
January	F11/08	Kidney	71,2	2,02	31,04	22,54
January	F11/08	Kidney	204,0	2,02	31,60	20,98
January	F11/08	Kidney	170,4	2,04	33,45	21,91
January	F11/08	Kidney	234,0	1,99	34,47	22,19
January	F21/08	Kidney	532,6	2,02	Neg.	20,62
January	F21/08	Kidney	237,5	2,02	Neg.	21,76
January	F21/08	Kidney	549,8	2,04	Neg.	21,18
January	F21/08	Kidney	348,2	1,99	32,75	21,36
January	F25/08	Kidney	266,6	2,06	Neg.	23,82
January	F25/08	Kidney	151,7	2,03	40,88	22,99
January	F25/08	Kidney	241,2	1,96	Neg.	24,31
January	F25/08	Kidney	337,8	2,03	41,79	23,20
April	F95/08	Kidney	132,0	2,05	Neg.	21,59
April	F95/08	Kidney	214,9	2,04	34,69	22,72
April	F95/08	Kidney	224,2	2,04	Neg.	22,40
May	F188/08	Kidney	391,6	2,02	Neg.	22,70
May	F188/08	Kidney	188,1	2,03	Neg.	21,96
May	F188/08	Kidney	535,7	2,03	Neg.	22,89
May	F188/08	Kidney	451,3	2,04	Neg.	23,25
June	F195/08	Kidney	299,0	2,07	29,93	22,01
June	F195/08	Kidney	210,0	2,08	36,38	21,31
June	F195/08	Kidney	135,7	2,08	30,89	21,80
June	F195/08	Kidney	157,9	2,08	24,31	20,99
June	F216/08	Kidney	152,8	1,98	29,40	24,34
June	F216/08	Kidney	96,6	1,99	37,78	23,60
June	F216/08	Kidney	289,5	2,02	41,15	24,09
June	F216/08	Kidney	264,7	2,00	27,88	23,99
August	F332/08	Kidney	65,5	2,00	32,10	24,24
August	F332/08	Kidney	111,3	1,84	34,01	24,73
August	F332/08	Kidney	108,3	1,92	29,63	23,19
August	F334/08	Kidney	48,6	2,09	27,01	26,09
August	F334/08	Kidney	51,1	2,09	27,17	24,79
August	F334/08	Kidney	40,6	2,11	34,45	25,72
August	F334/08	Kidney	61,0	1,81	29,56	25,74
September	F372/08	Kidney	166,1	2,00	35,41	23,04
September	F372/08	Kidney	98,4	2,00	26,14	21,93
September	F372/08	Kidney	113,2	2,02	24,22	22,42
September	F372/08	Kidney	52,1	2,09	33,87	22,88

Table 15 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PD 2009 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
April	F127/09	Kidney	111,7	2,02	Neg.	22,77
April	F127/09	Kidney	221,2	2,02	Neg.	22,75
April	F127/09	Kidney	149,2	1,99	44,27	22,39
April	F127/09	Kidney	138,9	2,01	Neg.	22,91
May	F193/09	Kidney	145,9	1,98	33,02	23,33
May	F193/09	Kidney	210,7	2,02	38,38	22,77
May	F193/09	Kidney	77,9	1,97	36,62	23,62
May	F193/09	Kidney	108,4	2,02	Neg.	23,63
June	F211/09	Kidney	186,9	1,96	Neg.	23,53
June	F211/09	Kidney	153,3	2,01	Neg.	25,29
June	F211/09	Kidney	246,1	2,02	Neg.	24,38
June	F211/09	Kidney	27,1	1,90	Neg.	25,83
July	F267/09	Kidney	128,5	1,96	Neg.	25,71
July	F267/09	Kidney	114,6	1,97	Neg.	25,17
July	F267/09	Kidney	52,9	1,96	30,27	25,16
July	F267/09	Kidney	142,9	2,01	33,99	24,96
July	F279/09	Kidney	71,7	2,05	32,28	21,73
July	F279/09	Kidney	122,6	2,05	36,48	21,95
July	F279/09	Kidney	123,5	1,99	37,94	21,65
July	F279/09	Kidney	118,4	2,00	27,99	22,15
July	F286/09	Kidney	87,5	1,99	42,98	22,11
July	F286/09	Kidney	52,0	2,04	Neg.	23,20
July	F286/09	Kidney	54,1	1,85	Neg.	23,31
July	F286/09	Kidney	22,0	1,76	Neg.	22,66
July	F298/09	Kidney	151,1	1,91	32,49	26,75
July	F298/09	Kidney	73,3	1,86	37,39	27,00
July	F298/09	Kidney	52,3	1,93	35,12	25,98
July	F298/09	Kidney	66,6	1,85	34,13	27,26
August	F308/09	Kidney	76,2	1,85	31,16	22,67
August	F308/09	Kidney	54,2	1,95	37,74	22,72
August	F308/09	Kidney	49,8	1,94	30,30	22,9
August	F308/09	Kidney	91,5	1,91	31,71	22,28
August	F309/09	Kidney	34,1	2,05	31,08	23,91
August	F309/09	Kidney	105,8	1,94	41,22	23,21
August	F309/09	Kidney	76,0	2,01	39,00	22,13
August	F309/09	Kidney	29,4	1,95	34,28	23,73

Table 16 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PD 2009 NVIH.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct- value
November	12641,1,1	Multiorgan	2110,0	2,07	37,59	21,09
November	12641,1,3	Multiorgan	258,3	2,07	39,89	21,64
November	12641,1,4	Multiorgan	441,7	2,06	38,83	20,65
November	12641,1,5	Multiorgan	389,3	2,06	Neg.	21,63
November	12641,1,6	Multiorgan	218,6	2,08	Neg.	22,15
November	12641,1,7	Multiorgan	588,7	2,10	Neg.	21,75
November	12641,1,10	Multiorgan	385,1	2,07	Neg.	21,13
November	12641,2,2	Multiorgan	425,8	2,00	Neg.	20,99
November	12641,2,3	Multiorgan	601,6	2,00	Neg.	21,30
November	12641,2,4	Multiorgan	1152,5	1,94	Neg.	19,68
November	12641,2,5	Multiorgan	580,8	2,05	Neg.	21,76
November	12641,2,6	Multiorgan	162,8	2,04	42,68	21,75
November	12641,2,7	Multiorgan	446,6	1,96	41,09	22,14
November	12641,2,8	Multiorgan	495,6	2,04	Neg.	21,82
November	12641,2,9	Multiorgan	161,9	2,06	Neg.	21,94
November	12641,2,11	Multiorgan	207,9	2,01	Neg.	21,01

Table 17 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). HSMI 2005 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct- value	EF1A Ct- value
February	F31/05	Kidney	696,2	2,01	34,16	21,28
February	F31/05	Kidney	1078,2	1,99	29,65	21,63
February	F31/05	Kidney	456,0	2,02	29,87	21,35
February	F31/05	Kidney	663,7	2,00	24,06	22,43
April	F128/05	Kidney	135,8	1,96	34,68	25,52
April	F128/05	Kidney	147,5	1,89	39,96	25,47
April	F128/05	Kidney	189,7	2,00	Neg.	25,42
April	F128/05	Kidney	321,2	1,99	Neg.	24,23
April	F131/05	Kidney	290,2	2,03	Neg.	23,57
April	F131/05	Kidney	497,1	1,87	43,53	23,10
April	F131/05	Kidney	267,6	2,06	Neg.	23,81
April	F131/05	Kidney	679,6	1,97	37,60	23,43
May	F135/05	Kidney	1059,4	2,01	Neg.	21,29
May	F135/05	Kidney	1021,0	2,00	33,83	23,14
May	F135/05	Kidney	631,6	1,99	34,72	23,04
May	F135/05	Kidney	756,7	1,98	34,36	22,91
June	F173/05	Kidney	119,7	1,97	Neg.	23,11
June	F173/05	Kidney	159,3	2,03	42,08	24,36
June	F173/05	Kidney	159,4	2,04	36,48	24,71
June	F173/05	Kidney	107,2	2,00	Neg.	23,07
June	F184/05	Kidney	218,9	1,99	29,07	25,48
June	F184/05	Kidney	303,8	1,97	29,47	23,85
June	F184/05	Kidney	357,5	1,97	32,98	24,69
June	F184/05	Kidney	549,4	1,92	Neg.	24,04
June	F188/05	Kidney	302,1	2,04	Neg.	24,27
June	F188/05	Kidney	434,6	2,02	40,35	24,39
June	F188/05	Kidney	368,9	2,04	38,46	24,68
June	F188/05	Kidney	280,7	2,05	36,10	24,42
June	F198/05	Kidney	273,4	1,96	30,55	23,32
June	F198/05	Kidney	260,2	2,03	Neg.	24,15
June	F198/05	Kidney	342,0	2,04	31,35	23,16
October	F319/05	Kidney	684,8	1,97	34,19	23,49
October	F319/05	Kidney	735,1	1,95	33,67	23,48
October	F319/05	Kidney	564,1	1,94	32,36	22,61
October	F319/05	Kidney	516,6	1,96	34,56	22,35

Table 18 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). HSMI 2008 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct- value	EF1A Ct- value
January	F23/08	Kidney	607,5	1,92	38,13	25,65
January	F23/08	Kidney	449,8	2,02	33,19	23,10
January	F23/08	Kidney	389,6	2,04	30,09	23,97
January	F23/08	Kidney	65,0	2,01	30,45	21,69
March	F83/08	Kidney	61,8	1,94	16,65	22,89
March	F83/08	Kidney	173,5	1,89	44,43	26,47
March	F83/08	Kidney	138,6	1,91	33,72	24,73
March	F83/08	Kidney	233,3	1,94	30,69	25,77
June	F259/08	Kidney	161,2	1,96	32,86	23,27
June	F259/08	Kidney	151,2	1,92	35,04	24,67
June	F259/08	Kidney	70,9	1,80	32,50	25,44
September	F340/08	Kidney	142,0	1,97	29,97	28,02
September	F340/08	Kidney	108,7	1,95	30,28	26,94
September	F340/08	Kidney	161,7	1,92	18,65	25,54
September	F373/08	Kidney	229,9	2,01	24,27	24,74
September	F373/08	Kidney	122,1	2,03	25,65	26,37
September	F373/08	Kidney	103,3	2,06	21,93	27,46
September	F373/08	Kidney	105,4	1,98	19,24	25,96
September	F375/08	Kidney	218,8	2,00	18,06	23,95
September	F375/08	Kidney	170,4	2,00	20,99	23,02
September	F375/08	Kidney	168,2	2,00	18,15	23,58
September	F375/08	Kidney	381,7	2,01	18,07	21,67
October	F417/08	Kidney	219,2	1,99	24,82	29,07
October	F417/08	Kidney	154,9	2,03	23,10	23,06
October	F417/08	Kidney	201,3	2,01	26,81	22,83
October	F417/08	Kidney	148,0	2,02	18,39	23,35
December	F474/08	Kidney	224,3	2,06	26,82	23,68
December	F474/08	Kidney	173,6	2,06	28,32	23,99
December	F474/08	Kidney	207,2	2,06	31,94	27,53
December	F474/08	Kidney	71,1	1,69	31,94	23,30

Table 19 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). HSMI 2009 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
February	F40/09	Kidney	170,6	1,93	28,30	21,57
February	F40/09	Kidney	200,7	2,02	24,26	21,73
February	F40/09	Kidney	160,9	2,04	31,78	22,72
February	F40/09	Kidney	144,2	2,09	27,36	23,05
March	F110/09	Kidney	49,4	2,08	31,82	25,19
March	F110/09	Kidney	67,1	2,08	27,77	23,52
March	F110/09	Kidney	66,2	2,08	30,46	23,49
March	F110/09	Kidney	54,9	2,07	31,68	23,23
April	F139/09	Kidney	131,5	2,03	34,61	23,12
April	F139/09	Kidney	110,4	2,03	Neg.	23,98
April	F139/09	Kidney	55,9	1,83	Neg.	25,39
April	F139/09	Kidney	154,7	2,40	37,21	24,63
April	F150/09	Kidney	342,5	2,03	Neg.	21,10
April	F150/09	Kidney	284,7	2,04	40,42	21,66
April	F150/09	Kidney	445,8	2,02	Neg.	22,50
April	F150/09	Kidney	305,6	2,02	37,69	20,96
May	F160/09	Kidney	171,0	2,05	Neg.	21,92
May	F160/09	Kidney	85,5	2,04	Neg.	22,62
May	F160/09	Kidney	251,1	1,93	Neg.	39,68
May	F160/09	Kidney	258,0	2,02	Neg.	22,37
May	F164/09	Kidney	12,6	2,02	Neg.	27,73
May	F164/09	Kidney	65,1	1,95	Neg.	24,35
May	F164/09	Kidney	47,6	1,93	Neg.	24,66
May	F164/09	Kidney	50,3	1,83	36,20	25,40
June	F200/09	Kidney	37,9	2,03	Neg.	25,63
June	F200/09	Kidney	39,5	2,03	37,55	23,88
June	F200/09	Kidney	63,0	1,89	Neg.	25,06
June	F200/09	Kidney	59,4	1,94	Neg.	22,67
June	F203/09	Kidney	70,3	2,05	38,25	23,11
June	F203/09	Kidney	150,0	1,75	Neg.	23,87
June	F203/09	Kidney	48,5	1,94	Neg.	26,90
June	F203/09	Kidney	58,9	2,09	42,28	23,58
June	F245/09	Kidney	143,0	2,07	Neg.	23,17
June	F245/09	Kidney	181,2	1,88	Neg.	23,70
June	F245/09	Kidney	33,8	2,14	Neg.	24,31
July	F275/09	Kidney	43,7	2,13	33,15	24,58
July	F275/09	Kidney	37,7	2,10	29,05	24,92
July	F275/09	Kidney	42,0	2,05	27,66	25,37

Table 20 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). HSMI 2009 NVIH.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
June	9692,1	Multiorgan	46,1	1,99	33,68	24,69
June	9692,2	Multiorgan	219,3	1,93	34,67	23,50
June	9692,3	Multiorgan	124,8	1,96	34,36	24,84
June	9692,4	Multiorgan	117,3	1,81	35,78	25,72
September	13643,1	Multiorgan	120,4	2,01	31,10	23,82
September	13645,1,1	Multiorgan	251,6	1,98	33,53	23,54
September	13645,2,1	Multiorgan	282,6	2,01	35,32	22,42
September	13649,1	Multiorgan	118,3	2,00	36,25	25,34
September	13650,1	Multiorgan	269,9	1,99	Neg.	22,63
September	14181,1,1,1	Multiorgan	123,0	2,00	Neg.	23,59
September	14181,1,2,1	Multiorgan	59,7	2,03	36,24	24,22
September	14182,1	Multiorgan	342,1	2,01	37,20	21,74
September	14193,1,1,1	Multiorgan	118,4	1,74	39,65	22,77
September	14183,1,2,1	Multiorgan	256,1	2,01	Neg.	22,20
September	14962,1,2,1	Multiorgan	287,1	2,00	Neg.	21,53
September	14962,1,3,1	Multiorgan	501,5	2,01	Neg.	20,91
September	14962,1,4,1	Multiorgan	446,2	2,04	41,00	21,43
September	15209,3,2,1	Multiorgan	338,1	2,03	Neg.	21,09
September	15209,3,3,1	Multiorgan	151,1	2,07	37,86	21,51
September	15209,3,4,1	Multiorgan	230,3	20,3	39,66	21,59
September	15209,3,5,1	Multiorgan	120,8	2,11	32,56	21,26
September	15209,3,6,1	Multiorgan	248,8	1,95	34,46	22,89
September	15209,3,7,1	Multiorgan	130,0	2,10	Neg.	21,26
September	15209,3,8,1	Multiorgan	218,0	2,06	Neg.	21,69
September	15209,3,8,1	Multiorgan	190,6	2,03	Neg.	21,81
October	16270,1,1,1	Multiorgan	100,7	1,92	38,68	24,24
October	16270,1,2,1	Multiorgan	128,4	1,83	36,75	24,87
October	16270,1,3,1	Multiorgan	72,6	1,91	34,82	24,88
October	16270,1,4,1	Multiorgan	59,6	1,95	33,57	23,15
October	16270,1,5,1	Multiorgan	15,3	1,91	34,82	27,01
November	18528,1	Multiorgan	31,2	2,04	33,84	24,67
November	18529,1,1,1	Multiorgan	6,1	2,02	36,28	28,69
November	18529,1,2,1	Multiorgan	8,4	2,09	35,71	26,57
November	18529,1,3,1	Multiorgan	37,5	2,02	34,15	24,57
November	19382,1(1-2)1	Multiorgan	110,0	1,97	Neg.	22,33
November	19382,1,3-5,1	Multiorgan	144,3	1,98	Neg.	22,78
November	19558,1,1,1	Multiorgan	101,2	1,80	Neg.	23,37
November	19558,1,2,1	Multiorgan	219,3	2,00	36,79	23,22
November	19559,1,1,1	Multiorgan	150,0	1,99	28,92	24,29
November	19559,1,2,1	Multiorgan	199,4	1,98	30,87	22,27
December	2045,1(1-2)1	Multiorgan	127,9	2,00	Neg.	22,21
December	2045,1(3-4)1	Multiorgan	423,5	2,02	29,59	21,90

Table 21 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). CMS 2005 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
January	F18/05	Kidney	227,3	1,98	Neg.	27,75
January	F18/05	Kidney	554,9	1,98	40,31	24,83
February	F29/05	Kidney	29,7	2,04	33,16	23,83
February	F29/05	Kidney	261,3	2,04	32,58	26,24
February	F29/05	Kidney	330,3	2,04	33,69	27,04
February	F29/05	Kidney	202,6	2,01	33,54	26,56
March	F83/05	Kidney	179,5	2,14	31,89	24,98
March	F83/05	Kidney	442,8	2,06	33,91	22,18
March	F83/05	Kidney	553,1	2,00	32,31	24,37
June	F192/05	Kidney	256,8	2,01	31,97	24,11
June	F192/05	Kidney	359,8	2,07	33,92	26,24
June	F192/05	Kidney	358,9	2,07	35,53	23,35
June	F192/05	Kidney	331,0	2,03	37,82	29,28
September	F293/05	Kidney	343,6	2,04	34,17	24,97
September	F293/05	Kidney	244,2	2,03	32,35	Neg.
October	F311/05	Kidney	380,2	2,02	38,77	24,81
October	F311/05	Kidney	670,0	2,02	33,00	26,58
October	F311/05	Kidney	215,6	1,91	Neg.	30,41
October	F313/05	Kidney	397,5	1,92	37,79	25,85
October	F313/05	Kidney	510,4	1,99	31,14	25,83
October	F327/05	Kidney	67,5	1,85	Neg.	29,51
October	F327/05	Kidney	62,3	2,77	34,58	26,65
October	F327/05	Kidney	437,8	2,00	Neg.	25,97
October	F327/05	Kidney	217,0	1,90	Neg.	28,31
November	F336/05	Kidney	258,1	1,95	36,23	27,66
November	F336/05	Kidney	351,2	2,04	40,68	26,27

Table 22 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). CMS 2008 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
January	F24/08	Kidney	304,7	2,08	32,83	23,93
January	F24/08	Kidney	440,4	2,03	32,98	23,45
February	F70/08	Kidney	574,4	1,99	35,47	24,23
February	F70/08	Kidney	354,3	2,05	32,94	23,62
February	F70/08	Kidney	668,8	2,00	34,78	22,87
March	F75/08	Kidney	459,7	2,03	34,11	22,71
March	F75/08	Kidney	256,7	2,03	38,00	23,93
March	F75/08	Kidney	213,3	1,96	34,40	25,52
March	F75/08	Kidney	564,2	1,96	33,70	23,62

Table 23 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). CMS 2009 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
February	F54/09	Kidney	460,8	2,04	35,63	24,07
February	F54/09	Kidney	77,9	1,89	30,80	24,79
March	F112/09	Kidney	140,5	1,97	35,68	25,19
March	F112/09	Kidney	180,6	1,99	34,66	24,40
March	F112/09	Kidney	139,0	1,97	33,02	25,05
March	F112/09	Kidney	331,0	2,05	34,20	23,30
March	F141/09	Kidney	72,6	1,96	31,04	25,97
July	F285/09	Kidney	301,9	2,05	36,74	23,33
July	F285/09	Kidney	297,6	2,03	32,37	23,46
July	F285/09	Kidney	311,7	2,03	30,81	23,44
July	F285/09	Kidney	288,4	2,02	31,98	23,14
September	F329/09	Kidney	142,5	1,98	34,27	24,49
September	F329/09	Kidney	105,1	2,05	33,25	23,54
September	F329/09	Kidney	125,2	1,79	31,73	24,12
December	F514/09	Kidney	156,2	2,00	33,71	23,51
December	F514/09	Kidney	150,4	1,92	27,92	24,11

Table 24 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). CMS 2009 NVIH.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
January	1537,1,1,1	Multiorgan	83,0	2,00	32,76	23,11
January	1537,1,2,1	Multiorgan	188,0	1,96	32,62	22,61
January	1537,1,3,1	Multiorgan	350,9	1,97	41,07	22,10
January	1537,1,4,1	Multiorgan	164,7	1,97	35,42	22,92
January	1537,1,5,1	Multiorgan	167,3	1,97	39,00	22,70
January	1579,1,1,1	Multiorgan	357,7	1,96	Neg.	21,84
January	1579,1,2,1	Multiorgan	530,1	1,96	Neg.	21,01
January	1579,1,3,1	Multiorgan	332,5	1,99	Neg.	23,29
March	4611,1,1,1	Multiorgan	255,4	1,93	Neg.	22,83
March	4611,1,2,1	Multiorgan	182,7	1,89	Neg.	22,71
April	6566,1,1,1	Multiorgan	166,3	1,97	Neg.	22,27
April	6566,1,2,1	Multiorgan	101,1	2,02	Neg.	22,80
April	6566,1,3,1	Multiorgan	420,4	1,97	Neg.	22,39
June	9685,1,1	Multiorgan	68,8	1,98	Neg.	24,41
June	9685,2,1	Multiorgan	80,0	1,97	Neg.	24,26
July	11214,1	Multiorgan	71,2	1,99	Neg.	22,31
August	11847,1,1	Multiorgan	42,8	1,94	Neg.	26,82
August	11847,2,1	Multiorgan	144,8	1,98	Neg.	22,82
August	11847,3,1	Multiorgan	356,2	1,97	Neg.	21,49
August	11847,4,1	Multiorgan	235,6	2,00	Neg.	21,90
August	11847,5,1	Multiorgan	26,5	2,09	Neg.	22,72
September	16985,1,1,1	Multiorgan	61,7	1,87	29,24	24,39
September	16985,1,2,1	Multiorgan	56,8	1,86	38,22	24,29
September	16985,1,3,1	Multiorgan	149,7	1,83	36,49	24,25
September	16985,1,5,1	Multiorgan	111,7	1,82	Neg.	24,20
October	17825,1	Multiorgan	44,1	1,88	Neg.	23,61
November	18986,1	Multiorgan	70,6	2,01	38,11	23,06

Table 25 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PGI 2005 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
January	F22/05	Kidney	419,7	2,02	27,38	25,75
January	F22/05	Kidney	285,2	2,05	25,84	24,87
January	F22/05	Kidney	995,2	2,02	25,75	25,36
April	F299/05	Kidney	330,5	2,03	28,95	23,47
June	F195/05	Kidney	263,3	1,92	Neg.	28,97
June	F195/05	Kidney	429,2	1,97	35,63	25,13
June	F195/05	Kidney	454,8	1,99	30,88	24,45
June	F195/05	Kidney	408,6	1,97	34,84	24,66
June	F282/05	Kidney	343,1	2,01	25,88	23,55
June	F282/05	Kidney	165,1	1,96	27,98	24,40
August	F264/05	Kidney	336,7	2,21	28,53	24,75
August	F264/05	Kidney	128,8	1,94	28,48	25,26
October	F325/05	Kidney	451,6	2,00	27,95	24,26
October	F325/05	Kidney	320,8	2,02	29,51	24,02
November	F340/05	Kidney	728,7	1,96	33,88	22,98
November	F340/05	Kidney	602,9	1,99	27,85	23,49
November	F340/05	Kidney	450,9	1,98	31,19	21,47
November	F340/05	Kidney	506,9	1,97	31,83	23,82
November	F344/05	Kidney	491,6	1,97	28,04	22,62
November	F344/05	Kidney	463,3	2,03	32,51	23,20
November	F344/05	Kidney	108,8	1,95	31,58	25,43
December	F371/05	Kidney	672,2	1,97	28,52	23,70
December	F371/05	Kidney	610,0	1,99	34,18	25,04

Table 26 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PGI 2008 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
January	F12/08	Kidney	236,8	1,99	26,49	22,75
January	F12/08	Kidney	323,8	2,05	24,74	23,16
January	F12/08	Kidney	1091,4	2,04	23,37	21,13
January	F12/08	Kidney	356,6	2,06	26,33	22,57
September	F343/08	Kidney	274,4	1,94	19,99	22,42
September	F343/08	Kidney	300,7	2,02	18,74	22,89
September	F343/08	Kidney	178,6	1,97	21,57	22,91
September	F343/08	Kidney	204,3	2,02	23,91	24,98
September	F345/08	Kidney	139,5	1,93	30,69	24,64
September	F345/08	Kidney	107,0	1,95	29,91	24,68
September	F345/08	Kidney	137,8	1,94	21,94	23,67
September	F345/08	Kidney	232,4	1,91	32,19	25,22
September	F356/08	Kidney	377,1	2,00	16,92	22,68
September	F356/08	Kidney	96,8	1,98	18,90	22,87
September	F356/08	Kidney	321,2	1,90	24,27	22,96
September	F356/08	Kidney	84,6	1,97	23,06	23,29
September	F360/08	Kidney	144,3	2,01	23,71	22,54
September	F360/08	Kidney	410,8	2,03	20,06	22,22
September	F360/08	Kidney	164,7	1,91	20,02	23,31
September	F360/08	Kidney	79,0	2,03	25,30	22,66
September	F374/08	Kidney	238,3	1,98	27,82	24,11
September	F374/08	Kidney	108,2	1,94	23,15	24,55
September	F374/08	Kidney	146,7	1,92	21,77	23,73
October	F419/08	Kidney	332,2	2,06	18,83	21,51
October	F419/08	Kidney	480,5	1,98	19,24	22,51
October	F419/08	Kidney	525,5	1,98	18,15	21,99
October	F419/08	Kidney	420,8	2,05	18,64	21,28

Table 27 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PGI 2009 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
Febryary	F61/09	Kidney	352,5	2,06	37,02	22,42
Febryary	F61/09	Kidney	333,5	2,06	31,27	Neg.
Febryary	F61/09	Kidney	259,3	2,01	26,95	22,13
Febryary	F61/09	Kidney	287,0	2,03	28,68	21,68
September	F357/09	Kidney	84,2	1,68	23,40	22,01
September	F357/09	Kidney	445,7	1,93	32,86	21,54
September	F357/09	Kidney	388,8	1,99	27,44	22,18
September	F387/09	Kidney	93,2	2,00	33,73	23,89
September	F387/09	Kidney	45,1	1,86	39,52	23,63
September	F387/09	Kidney	158,7	1,98	20,08	23,26
September	F387/09	Kidney	95,4	1,87	23,19	23,87
November	F449/09	Kidney	512,8	1,98	30,36	23,96
November	F449/09	Kidney	320,8	1,98	24,48	23,72
November	F449/09	Kidney	285,5	2,04	29,78	22,36
November	F449/09	Kidney	347,2	2,04	21,60	21,54
November	F453/09	Kidney	283,2	2,02	34,64	21,84
November	F453/09	Kidney	76,2	1,95	30,96	22,75
November	F453/09	Kidney	302,6	2,03	33,37	21,37
November	F453/09	Kidney	181,6	2,03	37,45	21,51
November	F478/09	Kidney	290,5	2,09	28,35	20,87
November	F478/09	Kidney	508,0	2,08	28,01	19,56
November	F478/09	Kidney	529,3	2,05	29,68	20,18
November	F478/09	Kidney	482,6	2,07	31,07	20,59
November	F482/09	Kidney	444,9	1,99	25,78	22,02
November	F482/09	Kidney	146,4	1,99	25,66	21,99

Table 28 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). PGI 2009 NVIH.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct-value	EF1A Ct-value
February	3480	Multiorgan	254,2	2,02	43,3	23,49
March	3623,1	Multiorgan	205,3	2,05	Neg.	22,36
March	3623,2	Multiorgan	82,2	2,02	Neg.	22,10
April	6981,1	Multiorgan	92,3	1,88	Neg.	23,53
April	6983,1,1,1	Multiorgan	497,0	2,07	Neg.	23,21
April	6983,1,2,1	Multiorgan	20,9	2,26	Neg.	25,13
April	6983,1,3,1	Multiorgan	176,2	1,89	Neg.	23,23
September	14574,2,1	Multiorgan	174,7	2,01	Neg.	22,03
September	14574,2,2	Multiorgan	124,7	2,00	Neg.	22,32
September	14574,3,1	Multiorgan	102,1	2,03	Neg.	22,34
September	14574,5,1	Multiorgan	107,9	2,02	Neg.	22,29
October	15833,1	Multiorgan	360,6	2,03	Neg.	21,71
October	15835,1	Multiorgan	621,9	1,92	Neg.	22,14
October	18989,1	Multiorgan	700,7	2,09	Neg.	20,28

Table 29 – Fish individual data, purification data (NAC and 260/208 ratio), Ct- values of targeting gene from *D. lepeophtherii* (NUC) and Ct- values from housekeeping gene (EF1A). Autumn disease 2008 NVIB.

Sampling month	Code	Organ	NAC	260/280-ratio	NUC Ct- value	EF1A Ct-value
September	F379/08	Kidney	358,1	2,03	26,92	23,41
September	F379/08	Kidney	237,5	2,03	17,86	23,41
September	F379/08	Kidney	237,1	2,04	21,06	23,38
September	F379/08	Kidney	400,4	2,02	22,87	22,08
September	F376/08	Kidney	110,9	1,93	16,52	24,26
September	F376/08	Kidney	272,0	1,97	18,47	23,01
September	F376/08	Kidney	265,8	1,92	17,25	22,55
September	F376/08	Kidney	273,9	1,92	16,91	23,12
September	F380/08	Kidney	649,0	2,00	26,93	23,34
September	F380/08	Kidney	377,1	2,02	25,28	24,41
September	F380/08	Kidney	384,2	2,04	27,13	22,78
September	F380/08	Kidney	414,1	2,01	26,35	23,45
September	F393/08	Kidney	616,3	1,97	16,98	24,28
September	F393/08	Kidney	478,9	1,91	18,95	23,03
September	F393/08	Kidney	294,2	1,98	17,10	22,62
September	F393/08	Kidney	464,3	1,97	18,49	23,78
October	F398/08	Kidney	282,9	2,02	26,18	23,53
October	F398/08	Kidney	215,7	1,99	27,28	23,71
October	F398/08	Kidney	468,4	1,96	29,32	23,00
October	F398/08	Kidney	488,4	1,93	27,45	23,02
October	F404/08	Kidney	411,8	1,99	23,63	22,45
October	F404/08	Kidney	290,2	1,99	19,67	22,84
October	F404/08	Kidney	505,0	1,95	17,42	22,52
October	F404/08	Kidney	900,4	1,95	34,19	25,70
October	F419/08	Kidney	347,3	2,06	22,88	21,91
October	F419/08	Kidney	418,8	1,98	15,79	21,95
October	F419/08	Kidney	452,9	1,98	24,05	22,02
October	F419/08	Kidney	276,6	2,05	16,09	22,46