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Executive Summary

An outbreak of Viral Haemorrhagic Septicaemia (VHS), as defined by European Community Council Directive 2006/88/EC, was detected during December 2012 in multiple species of wrasse (Labridae) stocked onto six sea-water sites around Shetland Mainland. The wrasse were originally captured from the wild off the west coast of the Scottish mainland and were being used as a biological control of sea-lice (Caligidae) on Atlantic salmon (*Salmo salar*) farms.

Inspection, diagnostic testing, contact tracing, epidemiological enquires and other relevant research were undertaken as part of an outbreak investigation, containment areas were established, and the removal of stocked wrasse was initiated. To date three of the six affected sites have been cleared with a substantial proportion (≈99%) of wrasse removed from the remaining sites.

Species other than wrasse were also tested for VHS. Lumpsucker (*Cyclopterus lumpus*) and Atlantic salmon stocked on VHS positive sites tested VHS negative. Wild poor cod (*Trisopterus minutus*) from within the pens of a VHS positive site tested VHS positive. Free-ranging wild Norway pout (*Trisopterus esmarkii*), sprat (*Sprattus sprattus*), grey gurnard (*Eutrigla gurnardus*), herring (*Clupea harengus*), whiting (*Merlangius merlangus*) and plaice (*Pleuronectes platessa*) from a locality around Shetland tested VHS positive.

A qualitative risk analysis suggests that the chance of this outbreak originating from the marine environment around Shetland is moderate and alternative possibilities are either low or negligible.

Abbreviations

CDN	Confirmed Designation Notice
CI	confidence interval
CPE	cytopathic effect
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
EC	European Community
ELISA	Enzyme-linked immunosorbent assay
EU	European Union
FHI	Fish Health Inspectorate
G-gene	glycoprotein gene
hap_D	haplotype of VHSV isolates from the first-reported site
ICTV	International Committee on Taxonomy of Viruses
IDN	Initial Designation Notice
IHN	Infectious Haematopoietic Necrosis
IHNV	*Infectious hematopoietic necrosis virus*
IPN	Infectious Pancreatic Necrosis
ISA	Infectious Salmon Anaemia
ISAV	*Infectious salmon anemia virus*
MS	Marine Scotland
MSS	Marine Scotland Science
N-gene	nucleocapsid gene
OIE	World Organisation for Animal Health
PCR	polymerase chain-reaction
qRT-PCR	quantitative reverse-transcriptase polymerase chain-reaction
RNA	ribonucleic acid
Se	diagnostic sensitivity
Sp	diagnostic specificity
VHS	Viral Haemorrhagic Septicaemia
VHSV	*Viral hemorrhagic septicemia virus*
VTM	viral transport medium
<	less than
>	greater than
≤	less than or equal to
≥	greater than or equal to
≈	approximately

Glossary of Technical Terms

Atlantic cod	*Gadus morhua* (Linnaeus, 1758)
Atlantic salmon	*Salmo salar* (Linnaeus, 1758)
ballan wrasse	*Labrus bergylta* (Ascanius, 1767)
common dab	*Limanda limanda* (Linnaeus, 1758)
Confirmed Designation Notice	a notification of restrictions on aquaculture production activities for a specified area, defined by the Aquatic Animal Health (Scotland) Regulations 2009 28 & 29, to prevent or limit the spread of a confirmed listed or emerging disease
corkwing wrasse	*Symphodus melops* (Linnaeus, 1758)
cuckoo wrasse	*Labrus mixtus* (Linnaeus, 1758)
Dover sole	*Solea solea* (Linnaeus, 1758)
enzyme-linked immunosorbent assay	a laboratory test that uses antibodies to detect the presence of a pathogen through a colour change
diagnostic testing	tests carried out on symptomatic individuals to establish the health status of a population with reference to specific pathogens
disease	clinical or non-clinical infection with one or more aetiological agents as defined in EC Council Directive 2006/88/EC
European seabass	*Dicentrarchus labrax* (Linnaeus, 1758)
facility	a site with the capability of carrying out tank-based research
farm	a commercial Atlantic salmon production site
fathead minnow	*Pimephales promelas* (Rafinesque, 1820)
first-reported site	the site at which VHS was first confirmed
fisherman	the individual contracted to catch wild wrasse for the fish-farm client
fish-farm client	the fish-farm company for which the wild-caught wrasse were captured, held, and deployed
flounder	*Platichthys flesus* (Linnaeus, 1758)

gadoid	species of the family Gadidae (Rafinesque, 1810)
genotype	a classificatory grouping of VHSV based on nucleic acid sequence variation
goldsinny wrasse	*Ctenolabrus rupestris* (Linnaeus, 1758)
grey gurnard	*Eutrigla gurnardus* (Linnaeus, 1758)
haddock	*Melanogrammus aeglefinus* (Linnaeus, 1758)
haplotype	isolates sharing a common nucleic acid sequence
herring	*Clupea harengus* (Linnaeus, 1758)
Initial Designation Notice	a notification of restrictions on aquaculture production activities for a specified area, defined by the Aquatic Animal Health (Scotland) Regulations 2009 24 & 25, to prevent or limit the spread of a suspected listed or emerging disease
Infectious Haematopoietic Necrosis	infection with *Infectious hematopoietic necrosis virus* (ICTV, 2000)
Infectious Pancreatic Necrosis	infection with *Infectious pancreatic necrosis virus* (ICTV, 1991)
Infectious Salmon Anaemia	infection with *Infectious salmon anemia virus* (ICTV, 2005)
lemon sole	*Microstomus kitt* (Walbaum, 1792)
long-rough dab	*Hippoglossoides platessoides* (Fabricius, 1780)
lumpsucker	*Cyclopterus lumpus* (Linneaus, 1758)
mackerel	*Scomber scombrus* (Linneaus, 1758)
Marine Scotland	the lead marine management organisation in Scotland which acts on behalf of the Scottish Ministers as the competent authority for fish, shellfish and crustacean diseases
Marine Scotland Science	a division of Marine Scotland incorporating predecessor Fisheries Research Services
Norway pout	*Trisopterus esmarkii* (Nileson, 1855)
nucleic acid	a biological molecule composed of DNA or RNA which encodes and transmits biological information

nucleotide	an individual component of nucleic acids
outbreak	an identified occurrence of infection involving one or more aquatic animals
plaice	*Pleuronectes platessa* (Linneaus, 1758)
pogge	*Agonus cataphractus* (Linneaus, 1758)
poor cod	*Trisopterus minutus* (Linneaus, 1758)
primer	a nucleic acid functioning as a starting position for DNA synthesis
rockcook wrasse	*Centrolabrus exoletus* (Linnaeus, 1758)
saithe	*Pollachius virens* (Linnaeus, 1758)
salmon louse	*Lepeophtheirus salmonis* (Krøyer, 1837)
salmon sealouse	*Caligus elongatus* (von Nordmann, 1832)
sea-lice	species of the family Caligidae (Brumeister, 1834)
short-horn sculpin	*Myoxocephalus scorpius* (Linnaeus, 1758)
Sound of Arisaig	the area of sea north of the Ardnamurchan peninsula through to the east coast of the island of Eigg and south of Arisaig
sprat	*Sprattus sprattus* (Linnaeus, 1758)
site	location of an aquaculture production business; further sub-classified as farms or facilities
statutory testing	tests carried out on targeted individuals to establish the health status of a population with reference to specific pathogens
temporary holding facility	a site to which wild-caught wrasse were moved following capture prior to onward transport
turbot	*Scophthalmus maximus* (Linnaeus, 1758)
Viral Haemorrhagic Septicaemia	infection with *Viral hemorrhagic septicemia virus* (ICTV, 2000)
whiting	*Merlangius merlangus* (Linnaeus, 1758)
wrasse	species of the family Labridae (Cuvier, 1816)

1 Introduction

Wrasse are a diverse family (Labridae) of marine fish comprising multiple genera occurring in tropical, subtropical and temperate seas[1]. Wrasse are increasingly used as a biological control of the salmon sealouse (*Lepeophtheirus salmonis)* and salmon louse (*Caligus elongatus*) on Atlantic salmon (*Salmo salar*) marine production sites (farms) and may be wild-caught or hatchery-reared[2].

Wrasse are known to be potentially susceptible to several bacterial, viral and parasitic pathogens[3] although there is no previous report of susceptibility to *Viral hemorrhagic septicemia virus*[4] (VHSV). This pathogen, a negative sense single-stranded RNA virus assigned to the taxonomic family *Rhabdoviridae* and genus *Novirhabdovirus*[5], is known to infect a wide variety of marine and freshwater fish species and has the potential to cause clinical disease in at least some species of salmonid[6]. Infections of VHSV are listed as a non-exotic disease under European Community (EC) Council Directive 2006/88/EC[7] and in Scotland are notifiable to the competent authority under the Aquatic Animal Health (Scotland) Regulations

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1 Parenti, P. and Randall, J.E. 2000. An annotated checklist of the species of labroid fish families Labridae and Scaridae. *Ichthyological Bulletin of the J.L.B. Smith Institute of Ichthyology*, **68**, 1-97.

2 Torrissen, O., Jones, S., Asche, F., Guttormsen, A., Skilbrei, O.T., Nilsen, F., Horsberg, T.E. and Jackson, D. 2013. Salmon lice – impact on wild salmonids and salmon aquaculture. *Journal of Fish Diseases*, **36**, 171-194.

3 Treasurer, J.W. 2012. Diseases of north European wrasse (Labridae) and possible interactions with cohabited farmed salmon, *Salmo salar* L. *Journal of Fish Diseases* **35**, 555-562.

4 The American English spelling of this virus, and others, is defined by the International Committee on Taxonomy of Viruses.

5 van Regenmortel, M.H.V., Fauquet, C.M., Bishop, D.H.L., Carstens, E.B., Estes, M.K., Lemon, S.M., Maniloff, J., Mayo, M.A., McGeoch, D.J., Pringle, C.R. and Wickner, R.B. 2000. *Virus Taxonomy*. San Diego, Academic Press.

6 Skall, H.F., Olesen, N.J. and Mellergaard, S. 2005. Viral haemorrhagic septicaemia virus in marine fish and its implications for fish farming – a review. *Journal of Fish Diseases*, **28**, 509-529.

7 Council Directive (EC) 2006/88/EC on animal health requirements for aquaculture animals and products thereof, and on the prevention and control of certain diseases in aquatic animals [2006]. *Official Journal of the European Union*, **L328**, 14-56.

(2009)[8]. Four major groups (genotypes) of VHSV named I to IV[9] are recognised by the World Organisation for Animal Health (OIE)[10].

This report describes the occurrence and control of an outbreak of VHS in Scotland commencing during 2012 in wild-caught wrasse used for the control of sea-lice on Atlantic salmon farms.

2 Chronology of the Outbreak

2.1 Notification of a Suspected Outbreak

Information regarding a suspected outbreak of VHS was received by Marine Scotland (MS) which acts on behalf of the Scottish Ministers as the competent authority for fish, shellfish and crustacean diseases on the 13 December 2012. The suspicions were based on increased mortalities and a commercially-sourced positive test result for wrasse held for a fish farming company (hereafter referred to as the fish-farm client) at a commercial tank-based facility in south-west Shetland mainland. An Initial Designation Notice (IDN) was served on the site under the Aquatic Animal Health (Scotland) Regulations 2009.

2.2 Confirmation of the Outbreak

The MS Fish Health Inspectorate (FHI) inspected the site on 15 December 2012. The facility was stocked with multiple marine species at the time of the inspection although none of these were listed as VHS susceptible under EC Council Directive 2006/88/EC as amended by European Union (EU) Commission Implementing Directive 2012/31/EU[11]. The wrasse consisted of approximately 10,000 wild-caught individuals comprising ballan wrasse (*Labrus bergylta*), corkwing wrasse (*Symphodus melops*), cuckoo wrasse (*Labrus mixtus*),

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8 The Aquatic Animal Health (Scotland) Regulations 2009 (S.S.I. 2009/85).

9 Snow, M., Cunningham, C.O., Melvin, W.T. and Kurath, G. 1999. Analysis of the nucleoprotein gene identifies distinct lineages of viral haemorrhagic septicaemia virus within the European marine environment. *Virus Research*, **63**, 35-44.

10 World Organisation for Animal Health 2012. On-line update of *Manual of Diagnostic Tests for Aquatic Animals*; sixth edition. Paris, OIE. Available at http://www.oie.int/international-standard-setting/aquatic-manual/access-online/ (accessed 31 May 2013).

11 Commission Implementing Directive (EU) 2012/31/EU amending Annex IV of Council Directive 2006/88/EC as regards the list of fish species susceptible to Viral haemorrhagic septicaemia and the deletion of the entry for Epizootic ulcerative syndrome [2012]. *Official Journal of the European Union*, **L297**, 26-28.

goldsinny wrasse (*Ctenolabrus rupestris*) and rockcook wrasse (*Centrolabrus exoletus*) on a diet of commercial dry pellets and cooked frozen pasteurised crab meat originating from the waters around Shetland. The FHI observed evidence of disease in the wrasse population and used targeted sampling to take tissues from a total of 40 individuals for diagnostic and statutory testing.

Samples for diagnostic testing, from 10 individuals, were subjected to pooled laboratory tests for VHS by virus isolation with enzyme-linked immunosorbent assay (ELISA) confirmation (section 5.2), for Infectious Pancreatic Necrosis (IPN) by virus isolation and for Infectious Salmon Anaemia (ISA) using the quantitative reverse-transcriptase polymerase chain-reaction (qRT-PCR). The two pools comprised five individuals of multiple wrasse species. Both pools tested positive for VHS and negative for both IPN and ISA. Tissues were also subjected to histological examination. Overall the most significant lesions were observed in the heart, where mild to severe myocardial necrosis, endocarditis and subendocardial haemorrhage were noted for seven individuals. In addition, all 10 fish showed marked infiltration of the pancreas, one showed renal interstitial haemorrhage, and two showed haemorrhage and congestion in the spleen. This pathology is not regarded as being definitive of VHS. Histopathology also revealed parasites and granulomatous lesions in different organs for a few of the individuals but these are not regarded as unusual observations for fish originating from the wild environment.

Samples for statutory testing, from 30 individuals, were subjected to individual laboratory tests for both Infectious Haematopoietic Necrosis (IHN) and VHS by virus isolation with ELISA confirmation and also VHS by qRT-PCR (section 5.3). All individuals tested negative for IHN. All 30 individuals tested positive for VHS by virus isolation with ELISA confirmation and 27 individuals tested positive for VHS by qRT-PCR. A subsequent genotype-specific qRT-PCR assay (section 5.3) assigned the isolates to VHSV genotype III.

It was concluded that an outbreak of VHS had occurred. A Confirmed Designation Notice (CDN) was served on the site on 20 December 2012 under the Aquatic Animal Health (Scotland) Regulations 2009. The United Kingdom (UK) Department of Environment Food and Rural Affairs then informed the European Commission's Animal Disease Notification System in accordance with

European Economic Community Council Directive 82/894/EEC[12]. This was the first site at which the VHS outbreak was detected and is referred to as the 'first-reported' site hereafter. This site is not necessarily the origin of the outbreak.

2.3 Contact Tracing

Contact tracing revealed that the first-reported site had received the wrasse as a single delivery by road on 24 October 2012 from a temporary holding facility located on the west coast of the Scottish Mainland. An IDN was served on the temporary holding facility on 14 December 2012. There had been no movements of stock from the first-reported site since the arrival of the wrasse.

2.4 Inspection of the Temporary Holding Facility

The FHI inspected the temporary holding facility on 15 December 2012. The site was stocked with multiple marine species at the time of inspection including Atlantic cod (*Gadus morhua*), haddock (*Melanogrammus aeglefinus*) and turbot (*Scophthalmus maximus*), which are listed as susceptible species under EC Council Directive 2006/88/EC. The facility had also introduced European seabass (*Dicentrarchus labrax*) from France during September 2012 which are considered as vectors of VHS under conditions described in Annex I of EC Commission Regulation 1251/2008[13]. This population originated from a compartment declared free of VHS and the site of origin is not listed as holding VHS susceptible species; the population therefore met the health certification requirements of the regulation. Almost all wild-caught wrasse captured during 2012 had been moved to other sites with the exception of individuals retained by the temporary holding facility as broodstock.

Tissue samples were taken for statutory testing from 70 turbot, 35 ballan wrasse, 25 European seabass, 15 goldsinny wrasse, three whiting (*Merlangius merlangus*) and two Dover sole (*Solea solea*). Four of the ballan wrasse were representatives of the wild-caught population captured during 2012 and retained

[12] Council Directive (EEC) 82/894/EEC on the notification of animal diseases within the Community [1982]. *Official Journal of the European Communities,* **L378**, 58-62.

[13] Commission Regulation (EC) No 1251/2008 of 12 December 2008 implementing Council Directive 2006/88/EC as regards conditions and certification requirements for the placing on the market and the import into the Community of aquaculture animals and products thereof and laying down a list of vector species [2008]. *Official Journal of the European Union*, **L337**, 41-75.

as broodstock. The samples were subject to individual laboratory tests for VHS by virus isolation with ELISA confirmation. All 150 individuals tested negative.

Further testing on 150 fish of each susceptible species was not carried out because it would have had a disproportionate effect on the business given the extensive testing of wrasse stocked on other sites which had passed through the facility (sections 2.5 & 2.6).

2.5 Initial Tests at Other Sites

Contact tracing revealed that wild-caught wrasse passing through the temporary holding facility had been stocked onto 16 commercial Atlantic salmon farms in addition to the first-reported site since the beginning of June 2012. These were located around Shetland mainland and the west coast of the Scottish mainland and included all of the sites receiving wild-caught wrasse passing through the temporary holding facility during 2012. One of the farms had harvested its Atlantic salmon and the wrasse euthanased prior to the known start of the outbreak. The temporary holding facility had also supplied hatchery-reared wrasse to another farm on the east coast of Harris. Initial Designation Notices were served on 16 farms (excluding the harvested farm) between 14-18 December 2012 inclusive.

Tissue samples were taken for statutory testing from 15 wrasse from each of 15 farms and four wrasse from one farm on 18-19 December 2012 inclusive. This sampling strategy was intended to provide further information on the origin of the outbreak. Individuals were tested for VHS by virus isolation with ELISA confirmation. Three farms around Shetland mainland tested VHS positive and CDN served on these on 8 January 2013.

2.6 Further Tests at Other Sites

Between 82 and 155 wrasse were sampled from each of 11 of the 13 farms remaining under IDN between 16 January and 12 February 2013 inclusive. Individuals were tested for VHS by virus isolation with ELISA confirmation. Two farms, both located off Shetland mainland, tested VHS positive. Confirmed Designation Notices were served on these farms on 24 January 2013.

The FHI were unable to take samples from two farms, both on the west coast of the Scottish mainland, because they had depopulated after initial testing. One of the depopulated farms had moved the wrasse to supplement those of a neighbouring farm following storm damage. The wrasse on this recipient farm had also passed through the temporary holding facility and were VHS negative at the initial test. The incoming wrasse were distributed between all the cages on the recipient farm and made up about half of the wrasse on this farm during further testing. The further testing on the recipient farm is, therefore, applicable to the original wrasse populations on both the depopulated and recipient farms. The other depopulated farm had harvested its Atlantic salmon and incinerated the wrasse at its shore-base. Any remaining wrasse transported amongst the Atlantic salmon to the processing plant were euthanased, macerated, ensiled, and incinerated off-site. The VHS status of this farm is, therefore, based on the test results of only 15 individuals from the initial testing, although all wrasse on the farm had subsequently been euthanased and disposed of.

A summary of the test results is presented in Table 1 on the following page. A total of six VHS positive sites around Shetland, comprising all the sites stocking wrasse in this area, were detected and served with CDN. Initial Designation Notices for all other sites were withdrawn on 28 February 2013.

3 Wrasse Species Testing VHS Positive

One or more individuals for each of the five wrasse species (ballan, corkwing, cuckoo, goldsinny and rockcook) tested VHS positive. No other wrasse species were stocked. The prevalence of VHS positive individuals for each species has not been estimated because the targeted nature of sampling is likely to result in biased estimates.

4 Geographical Distribution of VHS

All six sites stocking wrasse around Shetland were positive for VHS. The sites were in three disparate localities (Figure 1 on page 13) comprising south-west Shetland mainland (three sites), north-west Shetland Mainland (two sites), and east Shetland mainland (one site). All tested sites stocking wrasse out-with Shetland were negative for VHS (Figure 1). There appears to be a cluster of VHS positive sites around Shetland.

Table 1. Summary of VHS test results on individual fish on farms

Site[α]	Operator[β]	Species Additional to Wrasse Tested	Test Result[γ,δ]		Date of CDN
A	a	Atlantic salmon	wrasse	2/15	8 Jan 2013
B	a	Atlantic salmon	wrasse	4/15	8 Jan 2013
C	a	Atlantic salmon, poor cod, saithe, whiting	wrasse poor cod	74/150 2/58	24 Jan 2013
D	b	lumpsucker	wrasse	30/30	20 Dec 2012
E	a	Atlantic salmon	wrasse	4/15	8 Jan 2013
F	a	Atlantic salmon	wrasse	5/82	24 Jan 2013
VHS negative sites	a		wrasse	0/182[ε]	not applicable
	a		wrasse	0/15	
	a		wrasse	0/165	
	a		wrasse	0/165	
	a		wrasse	0/154	
	a		wrasse	0/165	
	a		wrasse	0/165	
	a		wrasse	0/165	
	a		wrasse	0/170	
	c		wrasse	0/165	
	d	Dover sole, seabass, turbot, whiting	wrasse	0/50	

[α] codes for VHS positive sites only are given;

[β] operator names are coded;

[γ] number positive/number individuals tested using virus isolation with ELISA confirmation;

[δ] test results are always given for wrasse and for other species only when a VHS positive individual was detected;

[ε] wrasse originated from two farms with approximately equal numbers tested from each (section 2.6).

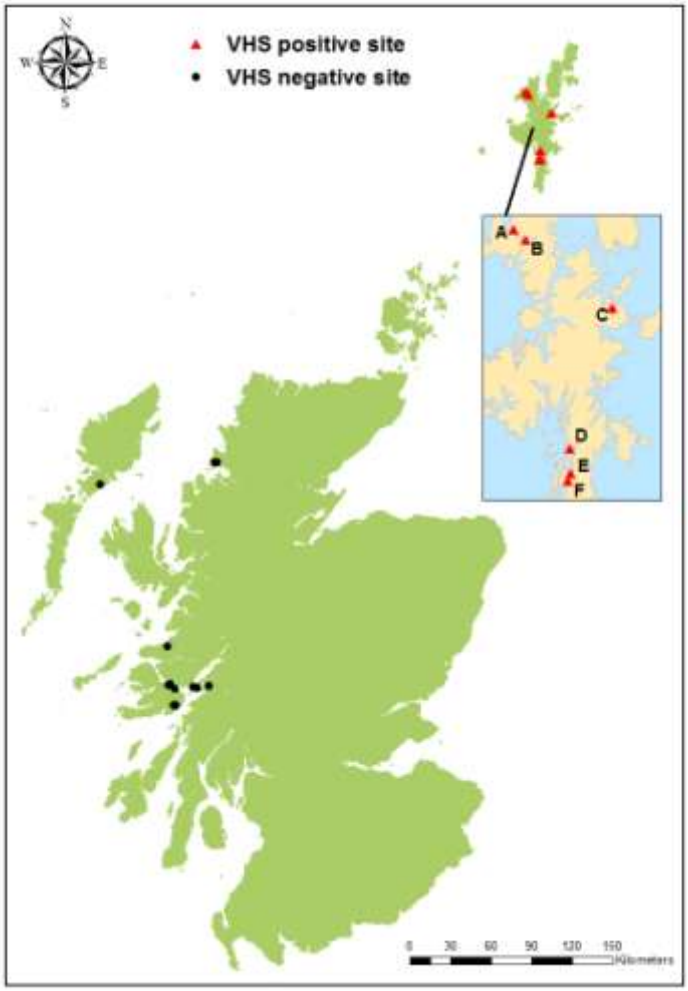

Figure 1. Location of VHS positive and negative sites

A spatial analysis utilising a Bernoulli model and a circular scanning window of variable radius, with p-values calculated by comparing the rank of the likelihood of the most likely cluster to the ranks of the likelihoods of datasets for which the VHS status of sites is randomised[14], was carried out using the specialist software SaTScan^{tm} (version 9.1.1)[15]. The most likely cluster, which includes all VHS positive sites around Shetland and no VHS positive sites elsewhere, can be regarded as statistically very highly significant ($p<0.001$). In conclusion, the cluster of VHS positive sites around Shetland has statistical support.

[14] Kulldorff, M. 1997. A spatial scan statistic. *Communications in Statistics – Theory and Methods,* **26**, 1481-1496.

[15] Kulldorff. M. and Information Management Services 2011. SaTScan™ v9.1.1; software for the spatial and space-time scan statistics. www.satscan.org (accessed 31 May 2013).

5 Laboratory Methods

5.1 Background

Marine Scotland Science (MSS) is a designated national reference laboratory for fish, shellfish and crustacean diseases in accordance with EC Council Directive 2006/88/EC. This includes accreditation (ISO 17020 and ISO 17025) and participation in ring-tests organised by the EU Reference Laboratory for Fish Diseases.

5.2 Virus Isolation with ELISA Confirmation

Virus isolation with ELISA confirmation as recommended by the OIE[10] was used to test individual wrasse for VHS on sites operating under IDN. The same procedure was used to test individual Dover sole, European seabass, turbot and whiting from the temporary holding facility operating under an IDN, individual Atlantic salmon on farms operating under CDN, individual wild gadoids in the pens of one farm operating under a CDN, and pooled free-ranging wild fish from south-west Shetland mainland.

Tissue comprising kidney, spleen, heart and brain from each sampled individual was placed in viral transport medium (VTM) and shipped to the laboratory in cool boxes containing frozen freezer blocks. Tissues were homogenised on receipt, centrifuged, the supernatant incubated with an equal volume of a laboratory-specific antiserum to *Infectious pancreatic necrosis virus* and inoculated onto a fathead minnow (*Pimephales promelas*) cell line[16] within 48 hours of sampling. The cells were incubated and inspected for a cytopathic effect (CPE) by phase-contrast microscopy three times a week and, in its absence after seven days, subcultured with continued inspection for a further seven days. A site for which no cultures showed CPE was declared VHS negative. Cultures showing CPE were further tested by ELISA using antibody MAb IP5B11[17]. A site for which the ELISA absorbance of one or more cultures exceeded a defined threshold value was declared VHS positive; otherwise the site was declared VHS negative.

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[16] Gravell, M. and Malsberger, R.G. 1965. A permanent cell line from the fathead minnow (*Pimephales promelas*). *Annals of the New York Academy of Sciences,* **126**, 555-565.

[17] Lorenzen, N., Olesen, N.J. and Jørgensen, P.E.V. 1988. Production and characterisation of monoclonal antibodies to four Egtved virus structural proteins. *Diseases of Aquatic Organisms,* **4**, 35-42.

The use of virus isolation with ELISA confirmation to test for VHS has not previously been subjected to a formal evaluation of its diagnostic specificity (Sp) and sensitivity (Se). Some information on the Sp of virus isolation with ELISA confirmation is available from the results of tests using the same procedure carried out at this laboratory in the five years prior to this outbreak. There was no outbreak of VHS in Scotland over this time and the methods, equipment and staff at the laboratory were relatively stable. None of the 293 VHS tests generated a positive result indicating a Sp for the laboratory procedure of greater than (>) 99% with a 95% confidence interval (CI) equal to or greater than (≥) 99%. There is insufficient laboratory data to estimate the Se of virus isolation with ELISA confirmation laboratory *per se*, although it is possible to estimate the probability of identifying a hypothetically infected farm out-with Shetland as being VHS positive[18]. Such an analysis involves assumptions including, for example, that the distribution of within farm infection prevalences on farms hypothetically infected with VHSV out-with Shetland would be the same as that observed on VHS positive farms around Shetland. It is concluded that there was a 99% (95% CI of 95% to >99%) chance of detecting a single farm out-with Shetland if it was hypothetically VHS positive.

5.3 Quantitative RT-PCR

Quantitative RT-PCR was used to screen individual wrasse and lumpsucker (*Cyclopterus lumpus*) at the first-reported site. Tissue comprising heart and kidney from each sampled individual was stored in the proprietary solution RNALater®. The test[19], developed by MSS, is a two-step procedure which detects a part of the nucleocapsid (N-) gene of VHSV genotypes I-IV.

[18] This involves fitting a beta-binomial distribution to the number of VHS positive wrasse and the number of wrasse tested on VHS positive farms around Shetland. This distribution is used to estimate the probability of each farm out-with Shetland testing VHS positive given the number of wrasse tested on those farms and assuming that the farms are hypothetically infected. The mean of these probabilities is the chance of detecting an individual farm out-with Shetland as VHS positive. Confidence intervals are obtained by subjecting the probabilities of detection to a bootstrap corrected for bias and acceleration.

[19] Matejusova, I., McKay, P., McBeath, A.J.A., Collet, B. and Snow, M. 2008. Development of a sensitive and controlled real-time RT-PCR assay for viral haemorrhagic septicaemia virus (VHSV) in marine salmonid aquaculture. *Diseases of Aquatic Organisms,* **80**, 137–144.

Quantitative RT-PCR was also used to determine the genotype of the VHSV infecting VHS positive wrasse at the first-reported site. The test[20], developed by MSS, categorises VHSV on the nucleic acid sequence variation of the N-gene enabling a rapid initial evaluation of the genotype and the risks to farmed and wild fish based on their relative susceptibility.

5.4 Nucleic Acid Sequencing

Partial nucleic acid sequences for the VHSV N- and glycoprotein (G-) genes were obtained for isolates propagated in cell culture. The isolates originated from four of the five species of wrasse and from all three localities around Shetland. Sequencing involved extracting RNA from VHSV infected cell culture material, conversion of RNA into complementary-DNA (cDNA), amplification of VHSV cDNA using the polymerase chain-reaction (PCR) with primers 5'ATGGAAGGA GGAATTCGTGAAGCG-3' and 5'GCGGTGAAGTGCTGCAGTTCCC-3' for the N-gene[21] and primers 5'-CATTTGTGCACACAACAAGCTAG-3' and 5'-GGTCATT CGGACAGGTGTGCTCAG-3' for the G-gene, agarose-gel purification of PCR products, and quantification of DNA prior to sequencing. The nucleic acid sequences were obtained using the amplification primers on a Beckman Coulter CEQTM 8800 Genetic Analysis System utilising the specialist software Sequencher® v4.9[22].

Nucleic acid sequence data for the N- and G-genes for a representative isolate of each of the four VHSV genotypes were obtained from the European Molecular Biology Laboratory nucleic acid sequence database[23]. These isolates[24] had

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[20] Bland, F., Snow, M., Garver, K.A. and Matejusova, I. 2013. Genotype-specific Taqman assays for the detection and rapid characterisation of European strains of viral haemorrhagic septicaemia virus. *Journal of Virological Methods*, **187**, 209-214.

[21] Snow, M., Bain, N., Black, J., Taupin, V., Cunningham, C.O., King, J.A., Skall, H.F. and Raynard R.S. 2004. Genetic population structure of marine viral haemorrhagic septicaemia virus (VHSV). *Diseases of Aquatic Organisms,* **61**, 11–21.

[22] Sequencher® version 4.9 sequence analysis software, Gene Codes Corporation, Ann Arbor, MI USA www.genecodes.com (accessed 31 May 2013).

[23] www.ebi.ac.uk/services/dna-rna (accessed 31 May 2013).

[24] The isolates comprised isolate DK-Hededam (Accession No. Z93412 for both N- and G-genes) as a representative of genotype I, isolate DK-1p53 (AJ130921 & AY546577 for N- and G- genes respectively) as a representative of genotype II, isolate UK-H17/2/95 (AJ130924 & AY546629 for N- and G-gene respectively) as a representative of genotype III and isolate US-Makah (X59241 & U28747 for N- and G-gene respectively) as a representative of genotype IV.

previously been used to demonstrate the utility of N- and G-gene nucleic acid sequence data for genotyping[25].

All sequences were aligned using the specialist software Clustal Omega[26] prior to statistical analysis, resulting in comparative nucleic acid sequences, containing no deletions or insertions, of 413 nucleotides from position 33 of the VHSV genome for the N-gene and 513 nucleotides from position 9134 of the VHSV genome for the G-gene.

6 Statistical Analysis

Statistical and modelling methods are described throughout. Unless stated otherwise analyses were carried out using the R statistical environment[27] (versions 2.14.1 and 2.15.2) utilising, as required, the supplementary R packages boot (version 1.3-7)[28], MASS[29] (version 7.3-22) and VGAM[30] (version 0.9-1).

7 Containment Areas Around VHS Positive Sites

Containment areas comprising an inner protection zone and an outer surveillance zone were established around the six sites operating under CDN in accordance with EC Council Directive 2006/88/EC.

Individual protection zones with a radius of 500 m were established around the five VHS positive farms. A smaller protection zone with a radius of 250 m was established around the first-reported site because there is disinfection of tank-effluent. The radius of the protection zones is supported by estimated pathogen

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[25] Einer-Jensen, K., Ahrens, P. and Lorenzen, N. 2005. Parallel phylogenetic analysis using the N, G, or Nv gene from a fixed group of VHSV isolates reveal the same overall genetic typing. *Diseases of Aquatic Organisms,* **67**, 39-45.

[26] Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., Lopez, R., McWilliam, H., Remmert, M., Söding, J., Thompson, J.D. and Higgins, D.G. 2011. Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular Systems Biology,* **7**(539), 1-6.

[27] R Core Development Team 2011 & 2012. R: a language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. ISBN 3-900051-07-0, www.R-project.org (accessed 31 May 2013).

[28] Canty, C. and Ripley, B. 2012. boot: bootstrap R (S-Plus) functions. R package version 1.3-7.

[29] Venables, W. N. and Ripley, B. D. 2002. *Modern Applied Statistics with S*. Fourth edn. New York, Springer.

[30] Yee, T.W. 2010. The VGAM package for categorical data analysis. *Journal of Statistical Software,* **32**(10), 1-34.

transmission distances using a transport-epidemiology model developed by MSS[31]. This model calculates the time required for the abundance of a pathogen released from a point source to decrease below its minimum infectious dose and then estimates the average distance that a peak amount of the pathogen travels over this time. Further details of the model and parameter values used are presented in Appendix 1.

Surveillance zones with a perimeter set at twice the radius of the protection zones have also been established. These zones do not include any aquaculture production facilities stocking fish other than those operating under CDN. Maps illustrating the containment areas for the six sites are presented in Figure 2 on the following page.

8 Removal of Wrasse from Sites

Wrasse at the first-reported site were euthanased, double-bagged and sent for incineration at the Lerwick waste management facility on 19 December 2012. The additional five VHS positive farms initiated a wrasse removal programme commencing 20-24 December 2012 inclusive. Stocked wrasse were captured using creels, raising the dead-basket, hand-nets and divers over a period of several weeks. Captured wrasse were euthanased and either incinerated at farm shore-bases with ash sent to landfill (farms on the west coast of Shetland mainland) or double-bagged and sent for incineration at the Lerwick waste management facility (the farm on the east coast of Shetland mainland). This progressive approach to removing wrasse was adopted to avoid unnecessary stress to Atlantic salmon stocks which are not listed as susceptible to VHS under EC Council Directive 2006/88/EC.

All wrasse are known to have been euthanased at the first-reported site. Two of the farms subsequently harvested their Atlantic salmon stocks during February and March 2013 with processing taking place at a plant located in south-west Shetland mainland in the same locality as three of the VHS positive sites. Three of the six sites have, therefore, been cleared of stocked wrasse.

[31] Salama, N.K.G. and Murray, A.G. 2011. Farm size as a factor in hydrodynamic transmission of pathogens in aquaculture fish production. *Aquaculture Environment Interactions,* **2**, 61 – 74.

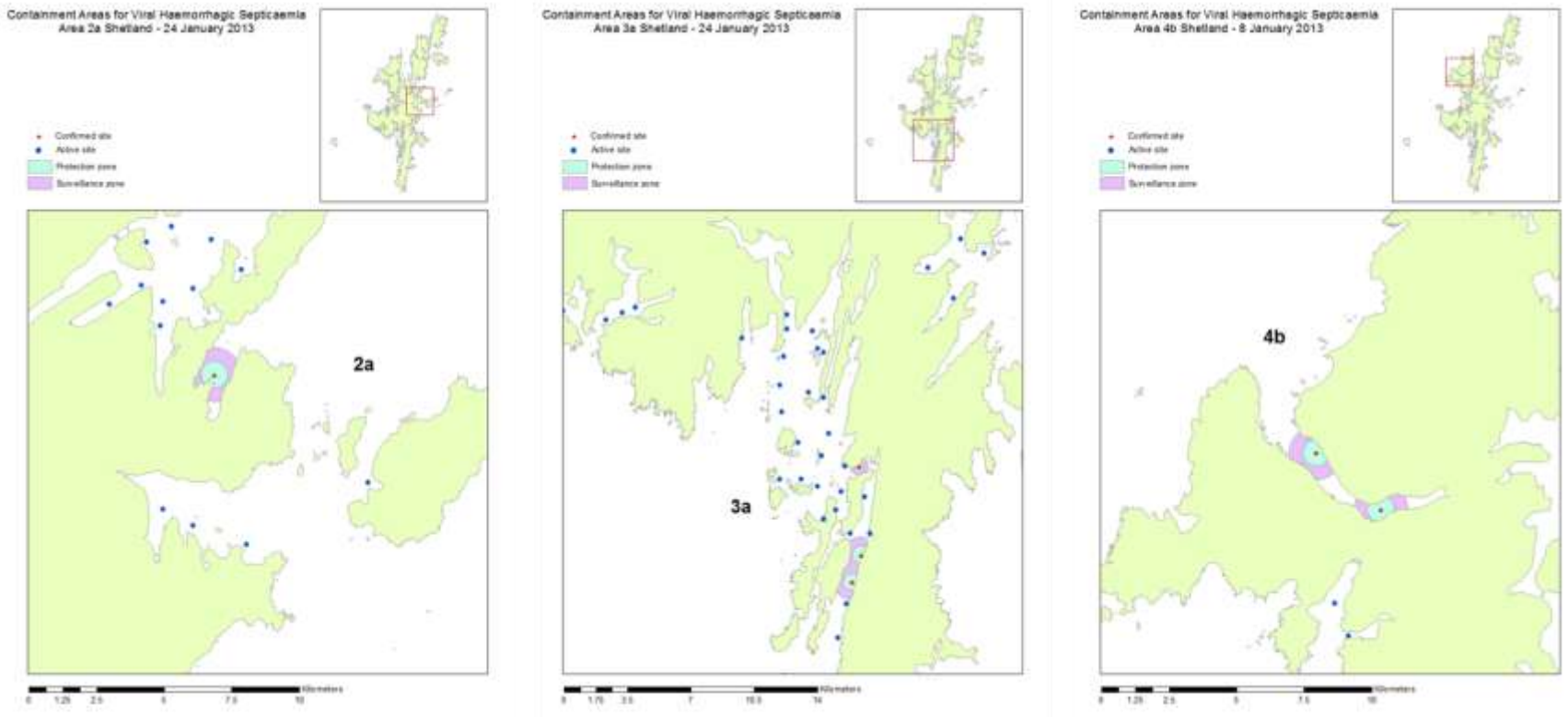

Figure 2. Containment areas around sites operating under CDN

Three farms remain in production with expected harvest dates of autumn 2013 and summer 2014. Records for these farms, covering a period of between eight to 11 weeks from the start of the wrasse removal programme, indicate that approximately 40,000 of an approximate 42,000 wrasse stocked onto the farms (95%) were either captured or described as mortalities. This estimate does not take into account undetected mortality prior to the start of the removal programme. Wrasse removal continues as wrasse are observed and the dead-basket is raised, and will continue, albeit with a reduced effort, until the farms are harvested and become fallow.

An alternative approach to evaluating wrasse removal is to fit a gamma distribution to the number captured each day and use this to estimate the proportion of wrasse removed by the end of the programme. Such an analysis involves assumptions including an equal effort to catch wrasse throughout the removal programme, but overcomes the problem of undetected mortality.

The results of such an analysis (Table 2) indicate that, overall, 99% of wrasse were removed. Output from the transport-epidemiology model (Appendix 1) indicates a reduction in the area around sites that the burden of VHSV exceeds the assumed minimum infectious dose.

Table 2. Removal of wrasse on three farms currently under production

Site	Farm Data			Model Output
	Initial Stock	**Recorded as Removed**[α]	**Removed** (%)	**Removed**[β] (%)
C	30,685	29,465	96%	≈ 99%
E	9,611	8,836	92%	≈ 99%
F	1,800	1,536	85%	> 99%
Overall	42,096	39,837	95%	≈ 99%

[α] including recorded mortality prior to the removal programme but not accounting for undetected mortality;

[β] assuming an equal effort to capture wrasse throughout the removal process.

9 Tests for VHS in Other Species

9.1 Atlantic Salmon on Farms

Five of the six VHS positive sites were Atlantic salmon farms. Atlantic salmon from each of these were tested for VHS even though the species is not listed as susceptible under EC Council Directive 2006/88/EC. This was done to check that the VHSV strain infecting wrasse had not become adapted to Atlantic salmon.

A total of 750 Atlantic salmon, consisting of 150 from each VHS positive farm, were individually tested using virus isolation with ELISA confirmation. This sample size is associated with a 95% chance of detecting at least one VHS positive individual at an apparent prevalence of 0.4%. All 750 individuals tested negative.

9.2 Gadoids Within Farm Pens

Wild gadoids were observed amongst Atlantic salmon in farm pens and some of the species are listed as susceptible under EC Council Directive 2006/88/EC.

A total of 63 gadoids, comprising 58 poor cod (*Trisopterus minutus*), four whiting and one saithe (*Pollachius virens*) representing all the populations of one VHS positive farm, were individually tested using virus isolation with ELISA confirmation. Two poor cod tested VHS positive. The apparent prevalence of VHS positive poor cod is three percent with a 95% CI of less than (<) one to 70%[32].

9.3 Lumpsucker at First-reported Site

A population of hatchery-reared lumpsucker supplied from Norway during November and December 2012 were held in the same room as the VHS positive wrasse at the first-reported site. The lumpsucker were screened for VHS even

[32] Gadoids in farm pens were sampled on two occasions (February and May 2013). One of 55 poor cod was VHS positive from the first sampling and one of three poor cod on the second sampling. Confidence limits were estimated by analysing the grouped data with a generalised linear model comprising an intercept only assuming a binomial error distribution and corrected for over-dispersion. The wide CI are a consequence of the over-dispersion between samplings.

though the species is not listed as susceptible under EC Council Directive 2006/88/EC. This was done because the lumpsucker were being held prior to deployment for an on-farm evaluation of their utility as a biological control for sea-lice and if infected could have represented a threat to the health of other aquatic animals.

A total of 630 lumpsucker were individually screened using qRT-PCR. This sample size is associated with a 95% chance of detecting at least one VHS positive individual at an apparent prevalence of 0.5%. All 630 individuals tested negative.

9.4 Free-ranging Wild Fish

Free-ranging marine fish were captured by demersal trawling around south-west Shetland mainland during April 2013. This locality was selected because it included the first-reported site and two of the VHS positive farms. Seven tows around the locality were carried out over four days (Figure 3 on the following page). Tissue samples from 1400 individuals were taken and grouped into 140 testing pools, each containing 10 individuals of the same species. The number of pools for each species were in approximate proportion to the species composition of individual tows. Further details of the sampling procedure are provided in Appendix 2.

A total of 11 pools of grey gurnard (*Eutrigla gurnardus*), herring (*Clupea harengus*), Norway pout (*Trisopterus esmarkii*), plaice (*Pleuronectes platessa*), sprat (*Sprattus sprattus*), and whiting were VHS positive by virus isolation with ELISA confirmation (Table 3 on page 24).

Eight percent of pools were VHS positive with 95% CI of four to 14%. This estimate is not a prevalence because the number of VHSV infected individuals within each pool is unknown. There is no evidence of a clustering of VHS positive pools by species or location of tow[33] although the statistical power of this comparison is likely to be low.

[33] Evaluated by comparing the magnitude of the variance of the number of VHS positive pools for each combination of species and tow with the expected variance estimated from multiple random assignments of VHS positive status to pools.

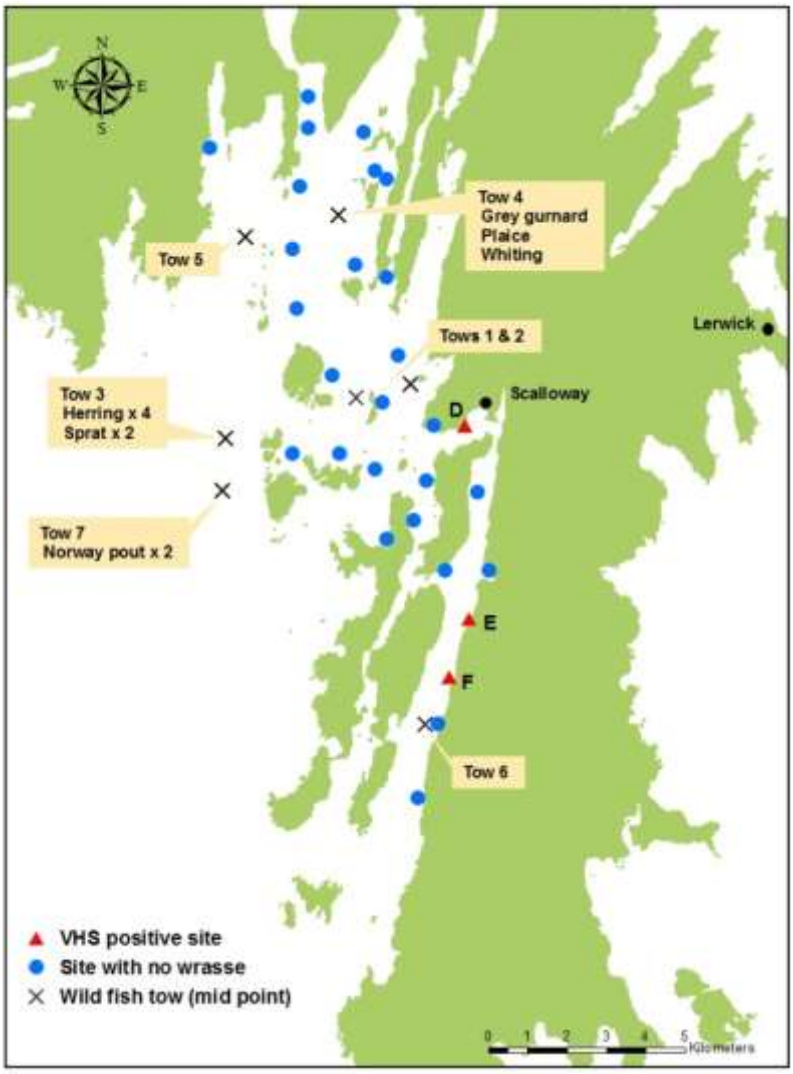

Figure 3. Location of wild fish sampling tows and VHS positive pools

10 Genetic Analysis

10.1 Background

Nucleic acid sequence variation between the VHSV isolates from this outbreak is less than or equal to (≤) one percent. This variation was used to assign isolates to haplotypes and to carry out a phylogenetic analysis using the specialist software PAUP* (version 4.0b10)[34].

[34] Swofford, D. L. 2003. PAUP*; Phylogenetic Analysis Using Parsimony (*and Other Methods). Version 4. Sunderland, Massachusetts, Sinauer Associates.

Table 3. Summary of VHS test results on pools of free-ranging wild fish

Species[α]	Test Result[β]
Norway pout (*Trisopterus esmarkii*)	2/4
sprat (*Sprattus sprattus*)	2/5
grey gurnard (*Eutrigla gurnardus*)	1/3
herring (*Clupea harengus*)	4/22
whiting (*Merlangius merlangus*)	1/24
plaice (*Pleuronectes platessa*)	1/40
common dab (*Limanda limanda*)	0/21
long-rough dab (*Hippoglossoides platessoides*)	0/5
haddock (*Melanogrammus aeglefinus*)	0/4
lemon sole (*Microstomus kitt*)	0/4
Atlantic cod (*Gadus morhua*)	0/3

[α] in addition one VHS negative pool for each of flounder (*Platichthys flesus*), mackerel (*Scomber scombrus*), pogge (*Agonus cataphractus*), saithe and short-horn sculpin (*Myoxocephalus scorpius*) were obtained but are not included in the body of the table;

[β] number positive/number pools tested using virus isolation with ELISA confirmation.

10.2 Confirmation of VHSV Genotype

Two viral isolates were recovered from corkwing and goldsinny wrasse at the first-reported site. The partial nucleic acid sequences for both isolates are the same and constitute a single haplotype designated, for the purposes of this report, hap_D.

The nucleic acid sequence of hap_D was used to confirm the initial assignment (section 2.2) of the outbreak to VHSV genotype III. Percentage dissimilarities between representative isolates (footnote 24) of each VHSV genotype and hap_D for the N- and G- genes are presented in Table 4 on the following page. The smallest difference is between hap_D and VHSV genotype III (1%). This similarity

was further confirmed by neighbor-joining[35]; the clustering of hap_D with VHSV genotype III is supported by a bootstrap value[36] approaching 100%. Analyses using other haplotypes from the other VHS positive sites involved in this outbreak generated the same result. This confirms that the virus responsible for the outbreak is VHSV genotype III.

Table 4. Nucleic acid sequence percentage dissimilarity between the haplotype from the first-reported site and VHSV genotypes

Isolate		Difference (%) for N-gene [α]				
		hap_D [γ]	**Gtp[δ]. I**	**Gtp. II**	**Gtp. III**	**Gtp. IV**
Difference (%) for G-gene [β]	**hap_D [γ]**		12	12	2	15
	Gtp[δ]. I	10		11	11	16
	Gtp. II	12	12		11	13
	Gtp. III	1	9	12		15
	Gtp. IV	16	14	16	16	

[α] 413 nucleotides compared;

[β] 513 nucleotides compared;

[γ] haplotype of isolates from the first-reported site;

[δ] a representative (footnote 24) of each VHSV genotype.

10.3 VHSV from Stocked Wrasse

Isolates with the same partial nucleic acid sequences as hap_D were recovered from rockcook wrasse from two VHS positive farms in north-west and east Shetland Mainland. A further three VHSV haplotypes (no codes assigned) were also identified from VHS positive wrasse.

Nucleic acid sequences for the four haplotypes were compared to the reference VHSV genotype III isolate UK-H17/2/95 which had been isolated from a haddock

[35] Saitou, N. and Nei, M. 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution,* **4**, 406-425.

[36] Felsenstein, J. 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution,* **39**, 783-791.

caught during a research cruise off eastern Scotland in 1995[37] as used to confirm the genotype of hap_D (section 10.2). The four outbreak haplotypes are more similar to each other (one to 10 nucleotide differences) than to the reference isolate (11 to 14 nucleotide differences).

The haplotypes were also subjected to a phylogenetic analysis using maximum parsimony[38,39]. The phylogenetic relationship between the reference isolate and the four outbreak haplotypes is undetermined with a bootstrap value of ≤ 50%. The four outbreak haplotypes cluster into two groups both supported by bootstrap values of ≥ 95% (Figure 4 on the following page). One group consists of hap_D and an isolate differing by two nucleotides recovered from rockcook wrasse on one of the two VHS positive farms in North-West Shetland mainland. The second group consists of two haplotypes differing from each other by a single nucleotide recovered from four species of wrasse (ballan, corkwing, goldsinny and rockcook) on two farms located close to each other in south-west Shetland; these two farms are closely linked because some stock on one farm was moved onto the other. The two groups of haplotypes differ from each other by seven to 10 nucleotides. These differences may be a consequence of either molecular evolution following a single infection event or of multiple infection events followed by more modest molecular evolution; there is insufficient data to resolve this.

10.4 VHSV from Gadoids Within Farm Pens

Isolates from two VHS positive poor cod on one of the farms (section 9.2) are assigned to haplotype hap_D, which is the same as isolates from two VHS positive rockcook wrasse stocked on the same farm. There is insufficient information to determine whether the poor cod contracted the infection from VHS positive

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[37] Smail, D.A. 2000. Isolation and identification of viral haemorrhagic septicaemia (VHS) viruses from cod *Gadus morhua* with the ulcus syndrome and haddock *Melanogrammus aeglefinus* having skin haemorrhages in the North Sea. *Diseases of Aquatic Organisms,* **41**, 231-235.

[38] Fitch, W. M. 1971. Toward defining the course of evolution: minimum change for a specified tree topology. *Systematic Zoology,* **20**, 406-416.

[39] The analysis comprised 1000 bootstrap replications of maximum parsimony branch-and-bound searches for the shortest unrooted tree using unweighted and unordered nucleic acid sequence differences of the N- and G-genes incorporated into the same analysis. Genotype III isolate UK-H17/2/95 was included for comparative purposes. While analyses utilising more sophisticated models are possible, the small number of substitutions available for analysis and uncertainty regarding the appropriate outgroup(s) suggests that this simple approach is the most suitable. The results of this analysis were confirmed using neighbor-joining.

wrasse on the farm or were representatives of the VHS positive free-ranging wild fish which infected the farm.

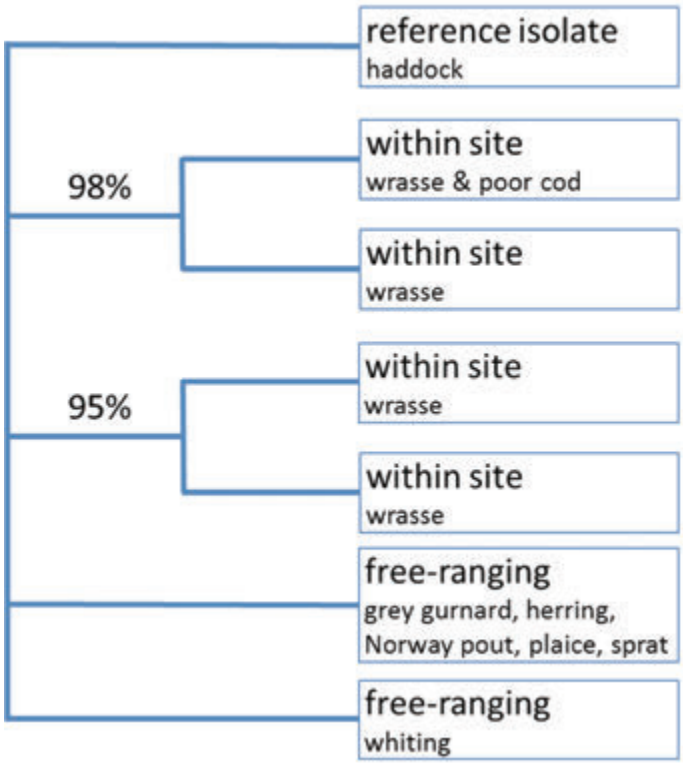

Figure 4. Maximum parsimony 50% majority rule consensus tree of phylogenetic relationships between haplotypes

10.5 VHSV from Free-ranging Wild Fish

Partial VHSV nucleic acid sequences were obtained from four pooled groups of herring, two pools of Norway pout, and one pool each of grey gurnard, plaice, sprat and whiting (section 9.4). Two haplotypes both of which differed from the VHSV isolates recovered from stocked wrasse (and poor cod within farm pens) were observed. The most frequent haplotype was common to all the wild fish species except whiting from which it differed by three nucleotides (<1%). The nucleic acid sequences are the consensus of VHSV from cell cultures of an unknown number of VHSV infected fish in each VHS positive pools and this constrains further inference regarding the amount of VHSV variation between VHS positive wild fish.

10.6 Comparison of VHSV Isolates Recovered from Sites and the Wild

The number of nucleotide sequence differences between VHSV haplotypes from stocked wrasse and wild species (three to nine nucleotides) is within the range of variation between haplotypes from stocked wrasse (one to 10 nucleotides). Nucleic acid sequences for the six haplotypes were subjected to a phylogenetic analysis using maximum parsimony as previously described[39]. The association between the wild fish and stocked wrasse haplotypes is uncertain (Figure 4) and more sequence data would be required to resolve this.

11 A 'History' of the VHS Positive Wrasse Populations

11.1 Capture

Free-ranging wild wrasse were caught by a single fisherman (hereafter referred to as 'the fisherman') contracted by the fish-farm client using a boat dedicated to this purpose. Wrasse were captured not less than 5 km from any active farm within an area north of the Ardnamurchan peninsula through to the east coast of the Isle of Eigg and south of Arisaig (hereafter referred to as the Sound of Arisaig) from June to October 2012 inclusive. Wrasse from this area were eventually stocked onto sites around Shetland mainland and the west coast of the Scottish mainland.

Wrasse were captured in baited creels deployed daily over approximately 500 m^2. Bait consisted of unpasteurised crustaceans caught within the area fished. Creels were emptied daily in the early morning and moved, without drying or disinfection, to another location within the same area. Species other than wrasse, described as cuddies (juvenile gadoids), conger eels and scorpion fish were also caught, but not in substantial numbers. These, together with wrasse which were regarded as being too small, were returned to sea during a sorting process.

Fish were sorted by species on the boat at the location of capture on a table connected by chutes to four sorting tanks. The wrasse from the sorting tanks were then counted into one of four on-board holding tanks. The water used for the tanks was from the area of catch and was not disinfected. Occasional mortalities (up to two) occurred during the approximate eight hours that the wrasse were on the boat. Tanks on the boat were occasionally steam cleaned.

11.2 Transport to the Temporary Holding Facility

Wild-caught wrasse destined for Shetland were transferred by road to the temporary holding facility in a transport tank on a pick-up by the fisherman (Figure 5). The transport tank and vehicle were steam cleaned after each delivery. The water used for the transport tank was from the point of loading and was not disinfected.

The same transport tank and vehicle were used to deliver wild-caught wrasse destined for sites out-with Shetland to the same temporary holding site. The transport tank and vehicle were also, on occasion, used to deliver wild-caught wrasse direct to farms rather than the temporary holding facility. The transport tank and vehicle were not used for any other type of delivery over this time.

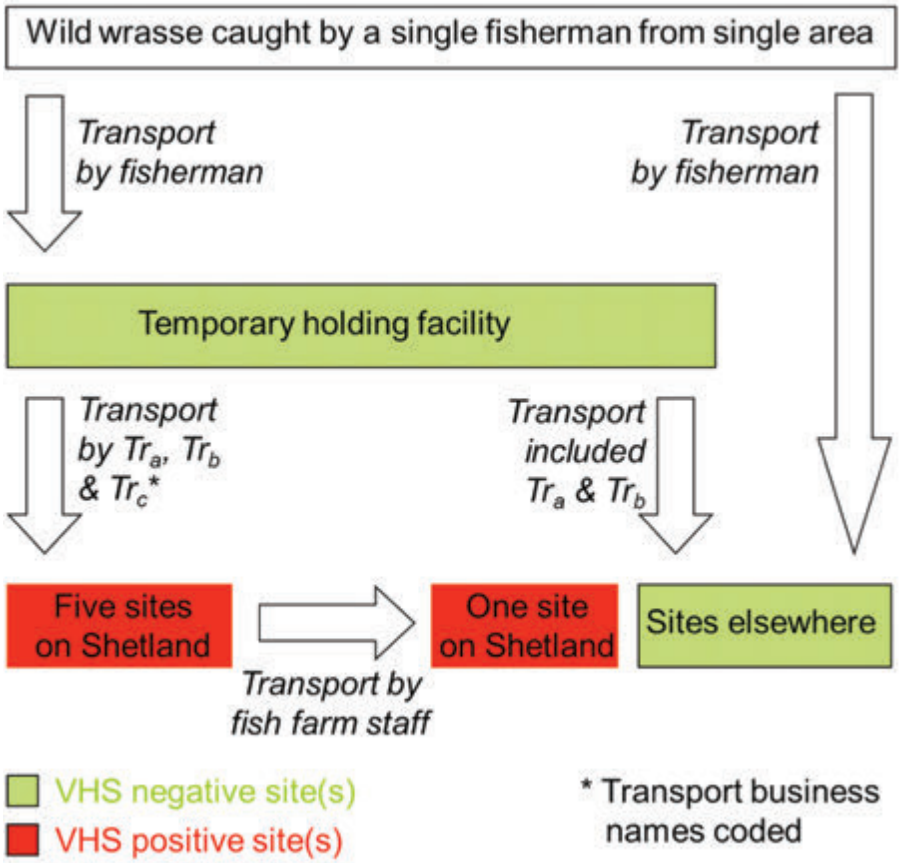

Figure 5. Movements of wild-caught wrasse stocked onto sites

11.3 Temporary Holding

The temporary holding facility is owned and operated by a business independent of the fisherman and fish-farm client. The temporary holding facility was contracted by the fish-farm client to hold, sequentially, four batches of 26,000 wild-caught wrasse prior to transport to Shetland for stocking on farms during 2012. The temporary holding facility also stocks broodstock and hatchery-reared wrasse and these were not supplied to Shetland.

Wild-caught wrasse intended for Shetland were brought onto the site on at least 26 occasions between 10 July and 21 October 2012 and held in up to five tanks located in three separate housed areas of the facility for varying periods of time. Incoming stock was added to existing stock with some splitting and merging of stock intended for Shetland.

The first two batches of wrasse for Shetland were subject to routine laboratory tests for VHS by a commercial provider during August 2012. Samples from a total of 120 individuals (60 for each batch) comprising mixed species of wild-caught wrasse were tested in pooled groups of five individuals using virus isolation with ELISA confirmation[40]. All pools tested negative for VHS.

In addition wrasse destined for farms operated by the fish-farm client out-with Shetland also passed through the temporary holding facility. At least three of the tanks, located across two areas on the facility, were used for these subsequent to their use for wrasse transported to Shetland.

Sea-water for the facility originates from an inlet located 0.8 km away from the outlet. Water supplying the site passes through a header-tank with removal of particulate matter through a sand-filter and disinfection by ultraviolet; there is no flow of water from tank-to-tank. All tanks were cleaned and disinfected on being emptied. While there was occasional shared use of some equipment such as hand nets and carrying bins, all but personal protective equipment was disinfected prior to moving. Footbaths were also present between areas of the site. Feed for wrasse destined for Shetland comprised commercial dry pellets.

[40] The detailed protocol of this virus isolation with ELISA confirmation test is not necessarily the same as that described in section 5.2 but was in accordance with OIE recommendations current at the time of testing.

In general the temporary holding facility attempted to keep stock for each of the four batches intended for Shetland as distinct populations with no carry-over of stock from batch to batch. In practice some carry-over of small numbers of fish may have occurred. Likewise, an attempt was made to keep stock intended for Shetland and elsewhere distinct with no carry over. While facility records are consistent with this, some carry over may have occurred on some occasions, although this is not certain.

11.4 Transport from the Temporary Holding Facility to Shetland

Wild-caught wrasse were transported from the temporary holding facility to five sites around Shetland mainland in nine shipments between 20 August and 31 October 2012 inclusive (Figure 5). Seven shipments were by one transport business (Tr_a) and one shipment each by another two businesses (Tr_b and Tr_c). The transport businesses were owned and operated independently of the fisherman, temporary holding facility and fish-farm client. Shipments involved disinfection of the tank, trailer and truck prior to entering the temporary holding facility, the loading of wrasse using disinfected water, transport by road and ferry to Lerwick, and transport by road to the shore-base of the destination sites. The wrasse from eight shipments were stocked onto single sites, and the wrasse from one shipment stocked onto two sites in the same locality sharing a shore-base.

The five sites received either one or two shipments by Tr_a. This transport business is a common link between VHS positive sites. The same trailer and tank was used for the deliveries although the truck itself was different. The trailer, tank and truck were disinfected prior to entering the temporary holding facility and following delivery. The trailer and tank were dedicated to the transport of wrasse and before commencing these deliveries had not been used since 2011. The transport business also made deliveries of wrasse from the temporary holding facility to multiple VHS negative sites out-with Shetland (Figure 5) and these were interspersed with deliveries to Shetland.

Two sites received a delivery by Tr_b. This transport business also made a delivery of wrasse to a VHS negative site out-with Shetland subsequent to this. The third transport business (Tr_c) did not make any subsequent wrasse deliveries.

11.5 Movements Within Shetland

One movement of wrasse within Shetland occurred (Figure 5). This involved the transfer of 1800 wrasse on 16 November 2012 from a farm which had received wrasse from the temporary holding facility on 6 September 2012 to a nearby farm sharing the same shore-base which had not (and did not) receive a delivery from the temporary holding facility. The movement was carried out by fish-farm staff.

12 Origin of the Outbreak

12.1 Background

Hypotheses regarding the origin of the outbreak are summarised and evaluated using information obtained from the outbreak investigation; only minimal reference is made to the scientific literature. The qualifiers used for the analysis are summarised in Table 5.

Table 5. Summary of qualifiers[41]

Qualifier	Interpretation
Negligible	Possible under exceptional circumstances
Low	Slight, but possible in some[α] circumstances
Moderate	Possible
High	A real possibility[β]

[α] translated into English from French as 'certain' in the source reference but modified for this report as 'some';

[β] interpreted for the purposes of this report as being beyond reasonable doubt.

[41] Zepeda-Sein, C. 1998. Méthode d'évaluation des risques zoosanitaires lors des échanges internationaux. In *Séminaire sur la Sécurité Zoosanitaire des Échanges dans les Caraibes*, 9-11 Décembre 1997, Port of Spain,(Trinidad and Tobago), 2-17, Paris, OIE. It has not been possible to obtain the original source and the version used is that given and translated in Dufour, B., Boisseleau, D., Chartier, C., Durand, B., Ganiere, J., Guillotin, J., Lancelot, R., Moutou, F., Saegerman, C., Thebault, A and Toma, B. 2008. A Qualitative Risk Assessment Method in Animal Health. Maisons-Alfort, Agence Francaise de Securite Sanitaire des Aliments.

12.2 Outbreak Originated from the Marine Environment of the Sound of Arisaig

Evidence supporting the hypothesis includes:

- the VHSV is genotype III (sections 2 & 10);
- VHS positive sites around Shetland received wrasse caught in this area (section 11).

Evidence contrary to the hypothesis includes:

- wild-caught wrasse for elsewhere were also captured from this area by the same fisherman but the outbreak was clustered around Shetland only (sections 4 & 11);
- tests for VHS on some of the wild-caught wrasse originating from this area, carried out while at the temporary holding site prior to the detection of the outbreak, were negative (section 11).

The chance that the outbreak originated from the marine environment of the Sound of Arisaig is categorised as 'low'. The reason for assigning a low category (rather than negligible) is that VHSV genotype III can be found in the marine environment and it is not possible to definitely determine that no VHS positive wild fish were present in the marine environment around the Sound of Arisaig prior to the outbreak.

12.3 Outbreak Originated During Transportation to the Temporary Holding Facility

Evidence supporting the hypothesis includes:

- VHS positive sites around Shetland received wild-caught wrasse which had been transported to the temporary holding facility (section 11).

Evidence contrary to the hypothesis includes:

- wild-caught wrasse destined for Shetland and elsewhere were transported to the temporary holding facility using the same transport tank and pickup but the outbreak clustered around Shetland only (sections 4 & 11);
- tests for VHS on some of the wild-caught wrasse transported to the temporary holding facility, carried out prior to the detection of the outbreak, were negative (section 11).

The chance that the outbreak originated during transport to the temporary holding facility is categorised as 'negligible'.

12.4 Outbreak Originated at the Temporary Holding Facility

Evidence supporting the hypothesis includes:

- VHS positive sites around Shetland received wild-caught wrasse which had been held at the temporary holding facility (sections 2 & 11);
- the temporary holding facility held VHS susceptible species at the time of the outbreak (section 2).

Evidence contrary to the hypothesis includes:

- wild-caught wrasse destined for Shetland and elsewhere were all held at the temporary holding facility but the outbreak clustered around Shetland only (sections 4 & 11);
- tests for VHS on some of the wild-caught wrasse at the temporary holding facility, carried out prior to the detection of the outbreak, were negative (section 11);
- tests for VHS on stocks other than-wild caught wrasse at or originating from the facility, carried out subsequent to the detection of the outbreak, were negative (section 2).

The chance that the outbreak originated at the temporary holding facility is categorised as 'low'. The reason for assigning a low category (rather than negligible) is that each susceptible species at the facility would have required testing at the 150 fish level for increased certainty.

12.5 Outbreak Originated During Transportation from the Temporary Holding Facility

Evidence supporting the hypothesis includes:

- VHS positive sites around Shetland received wild-caught wrasse which had been transported from the temporary holding facility (sections 2 & 11).

Evidence contrary to the hypothesis includes:

- the transport businesses disinfected transport tanks, trailers and trucks prior to entering the temporary holding site and used disinfected water from the temporary holding site (section 11);
- transport businesses Tr_a and Tr_b made subsequent deliveries of wild-caught wrasse to VHS negative sites out-with Shetland (sections 11);

- transport businesses Tr_b and Tr_c made only one delivery to single sites on Shetland but the outbreak involved multiple disparate localities around Shetland (section 11).

The chance that the outbreak originated during transport from the temporary holding facility is categorised as ‘negligible’.

12.6 Outbreak Originated at the First-Reported Site

Evidence supporting the hypothesis includes:

- wild-caught wrasse at the first-reported site were VHS positive (section 2).

Evidence contrary to the hypothesis includes:

- no species listed as susceptible under EC Council Directive 2006/88/EC were held at the facility while the wild-caught wrasse were on site (section 2);
- wrasse were fed a commercial dry pellet and cooked frozen pasteurised crab meat (section 2);
- tests for VHS, carried out subsequent to the detection of the outbreak, on 630 lumpsucker held in the same building as the VHS positive wrasse were negative (section 9);
- disinfection of effluent discharges was in-place;
- despite investigation no credible epidemiological link between the first-reported site and other VHS positive sites has been discovered.

The chance that the outbreak originated at the first-reported site is categorised as ‘negligible’.

12.7 Outbreak Originated from Atlantic Salmon Stocked on Site

Evidence supporting the hypothesis includes:

- wrasse testing VHS positive during the outbreak investigation were stocked with Atlantic salmon (section 2).

Evidence contrary to the hypothesis includes:

- all of 750 Atlantic salmon from VHS positive farms tested VHS negative (section 9);
- at least two independent batches of Atlantic salmon smolts had been split to stock VHS positive farms on Shetland and VHS negative farms elsewhere;

- all Atlantic salmon smolts originated from a continental zone of a member state declared free of VHS under EC Commission Decision 2009/177/EC[42] as amended by EU Commission Decision 2010/171/EU[43].

The chance that the outbreak originated from stocked Atlantic salmon is categorised as 'negligible'.

12.8 Outbreak Originated Directly from White Fish Processing Activities

Evidence supporting the hypothesis includes:

- VHS has been reported in wild commercial white fish species in the North-East Atlantic Ocean and North Sea[37,44,45];
- white fish processing takes place in one of the VHS positive localities.

Evidence contrary to the hypothesis includes:

- white fish processing does not take place in the other two VHS positive localities;
- the distances of VHS positive sites from white fish processors exceed the estimated transmission distance for VHSV (Appendix 1).

The chance that the outbreak originated directly from white fish processing activities is categorised as 'negligible'.

12.9 Outbreak Originated from the Marine Environment Around Shetland

Evidence supporting the hypothesis includes:

- the VHSV is genotype III (sections 2 & 10);
- the outbreak clustered around Shetland only (section 4);

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[42] Commission Decision of 31 October 2008 implementing Council Directive 2006/88/EC as regards surveillance and eradication programmes and disease-free status of Member States, zones and compartments. *Official Journal of the European Union*, **L63**, 15-39.

[43] Commission Decision of 22 March 2010 amending Annex I to Decision 2009/177/EC as regards surveillance programmes for Ireland and Hungary and the disease-free status of Ireland for certain aquatic animal diseases. *Official Journal of the European Union*, **L75**, 28-32.

[44] Smail, D.A. 1995. Isolation and identification of viral haemorrhagic septicaemia (VHS) virus from North Sea cod (*Gadus morhua* L.). *International Council for the Exploration of the Sea, Mariculture Committee*, CM 1995/F:15.

[45] King, J.A., Snow, M., Smail, D.A. and Raynard, R.S. 2001. Distribution of viral haemorrhagic septicaemia virus in wild fish species of the North Sea, North East Atlantic Ocean and Irish Sea. *Diseases of Aquatic Organisms*, **47**, 81-86.

- six species of free-ranging wild fish around south-west Shetland tested VHS positive (section 9);
- the first-reported site did not use disinfected seawater and was open to infection. The intake was located adjacent to the facility's outlet which was conducive to attracting wild fish thereby creating a potential route of infection into the facility consistent with the estimated transmission distance for VHSV (Appendix 1). The additional five VHS positive farms used net-pens which are open to infection.

Evidence contrary to the hypothesis includes:

- uncertainty regarding whether VHSV infections in free-ranging wild fish are the origin or a consequence of the outbreak;
- the involvement of multiple disparate localities around Shetland which, together with the limited estimated transmission distance for VHSV, requires either multiple infection events by wild fish or anthropogenic spread (section 4, Appendix 1).

The chance that the outbreak originated from the marine environment around Shetland is categorised as 'moderate'. The reason for assigning a moderate category (rather than high) is that the available evidence is not sufficiently unambiguous as to be able to state beyond reasonable doubt that this hypothesis is correct.

12.10 Conclusion

The most likely explanation of the origin of this outbreak is the marine environment around Shetland. The VHSV genotype III is recognised as being associated with the area of the North Atlantic to the Norwegian coast and the North Sea[10] and VHSV has previously been reported in free-ranging marine species in the vicinity of Shetland[44,45]. Further investigations focussing on how the risk of VHS affects the use of wrasse and other taxa as biological controls for sea-lice, and how the risks can be ameliorated, is desirable.

13 Acknowledgments

Disease outbreaks require a rapid, thorough and integrated response based on specialist experience to ensure effective control. Control of this outbreak has involved the combined efforts of the Fish Health Inspectorate, and the Diagnostic, Research and Epidemiology groups working within the infrastructure of MSS. Control of the outbreak would have been very much more difficult without the willing cooperation of affected (and potentially affected) aquaculture companies and staff.

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Appendix 1

Modelling the Dispersion of VHSV

N.K.G. Salama

To determine the potential transmission distances of VHSV from infected fish a coupled simplified transport-epidemiology model is used[31,46]. The model accounts for the biomass of infectious individuals at a site, the biomass of a potentially exposed site, the mean transmissibility rate of a pathogen between infected and susceptible individuals, the mean expression probability of an infected individual, the mean recovery probability of infected individual, the mean shedding rate of an infected individual, the minimum infective dose of a pathogen required to cause an infection to occur in a susceptible individual, the disease agent decay rate, the diffusion coefficient of the disease agent in the water, the tidal current amplitude, residual current speeds and tidal period. The model calculates the time until the pathogen abundance in the environment decreases below the minimum infectious dose. The model then estimates the average distance that the peak amount of shed pathogen particles can travel in this calculated time.

It is difficult to obtain the actual parameters that may occur for this current strain of VHSV and its interaction with wrasse species, as the strain characteristics are yet to be documented. Furthermore, research is limited as wrasse species had not been described as being susceptible to marine VHSV. As such, many of the parameters have to be approximated for other strains of VHSV with non-wrasse susceptible marine species, and also characteristics of other marine pathogens such as *Infectious salmon anemia virus* (ISAV) infecting salmonid hosts and the related Rhabdoviridae species *Infectious hematopoietic necrosis virus* (IHNV). The parameter values implemented in the model are described in Table 6 on the following page.

[46] Salama, N.K.G. and Murray A.G. 2013. A comparison of modelling approaches to assess the transmission of pathogens between Scottish fish farms: the role of hydrodynamics and site biomass. *Preventive Veterinary Medicine*, **108**, 285-293.

Table 6. Parameter values used for the VHSV transport-epidemiology model

Parameter	Value	Note on Source Value
Transmission	0.015 d^{-1}	as for ISAV in salmon[47]
Expression	0.14 d^{-1}	as for ISAV in salmon[47]
Recovery	0.04 d^{-1}	as for ISAV in salmon[47]
Shedding	10^3 pfu mL^{-1}	as for IHNV in salmon and within range of VHSV IVb in muskellunge[48,49]
Min. infective dose	10^2 pfu mL^{-1}	as for VHSV in Pacific herring[50]
Decay rate	0.06 h^{-1}	within range for North American strains in seawater[51,52]
Diffusion coefficient	10^4 cm^2 s^{-1}	within range for Scottish waters and previously used in model development[53]
Residual current speed	4cm s^{-1}	lowest recorded range observed between October 2004-2005 offshore eastern Shetland. For simplification, although unlikely, advection occurs in all directions.[54]
Tidal period	12.42 h	footnote 55
Tidal current amplitude	0.255 m s^{-1}	published approximation for Shetland[55]

[47] Gregory, A., Munro, L.A., Snow, M., Urquhart, K.L., Murray, A.G. and Raynard R.S. 2008. An experimental investigation on aspects of Infectious salmon anaemia virus (ISAV) infection dynamics in seawater Atlantic salmon, *Salmo salar* L. *Journal of Fish Diseases*, **32**, 481-489.

[48] Traxler, G.S., Roome, J.R. and Kent, M.L. 1993. Transmission of Infectious hematopoietic necrosis virus in seawater. *Diseases of Aquatic Organisms*, **16**, 111-114.

[49] Kim, R.K. and Faisal, M. 2012. Shedding of Viral haemorrhagic septicaemia virus (genotype IVb) by experimentally infected Muskellunge (*Esoc masquinongy*). *The Journal of Microbiology*, **50**, 278–284.

[50] Kocan, R., Bradley, M., Elder, N., Meyers, T., Batts, W. and Winton, J. 1997. North American strain of Viral haemorrhagic septicaemia virus is highly pathogenic for laboratory-reared Pacific herring. *Journal of Aquatic Animal Health*, **9**, 279–290.

[51] Kocan, R.M., Hershberger, P.K. and Elder, N.E. 2001. Survival of the North American strain of Viral haemorrhagic septicemia virus (VHSV) in filtered seawater and seawater containing ovarian fluid, crude oil, and serum-enriched culture medium. *Diseases of Aquatic Organisms*, **44**, 75-78.

[52] Hawley, L.M. and Garver, K.A. 2008. Stability of Viral haemorrhagic septicaemia virus (VHSV) in freshwater and seawater at various temperatures. *Diseases of Aquatic Organisms*, **82**, 171–178.

[53] Turrell, W.R. 1990. Simulation of advection and diffusion of released treatments in Scottish sea lochs. Scottish Fisheries Working Paper No. 16/90.

[54] Hughes, S.L, Beaton, J. and Slesser, G. 2006. Current meter measurements east of Shetland October 2004-October 2005: monitoring oceanic exchanges with the North Sea – data report. Fisheries Research Service Internal Report No. 10/06.

[55] Scottish Executive 2000. Final report of the joint government/industry working group on infectious salmon anaemia (ISA). Edinburgh, Scottish Executive.

The host number of infectious individuals is obtained by the biomass of wrasse recorded by MS FHI for each confirmed site. As the prevalence had yet to be determined, simulations were undertaken for each site with prevalence assigned within the range between 10–100%, increasing in 10% increments. The exposed site was taken to be the nearest site by Euclidian distance of sites held in the MS viewfarms GIS database. The biomass of this site was obtained from the records held in the MS FHI Aquadat database.

Although the model cannot be validated, using parameter values specific for ISAV and applying the physical characteristics described in Table 6 and using farm biomass values characteristic for the Scottish industry, it is possible to produce transmission distances in a similar scale to those designated for disease management areas for the control of ISA[31]. This indicates that the model is able to capture features which have proved useful in restricting the spread of ISA[56].

Implementing the parameters in to the model expression[46] and undertaking multiple simulations for varying prevalence, and biomass at each positive farm site and incorporating the biomass of salmon on the nearest neighbour it is possible to estimate that a transmission radius occurring within the range of around 400 m for farms with possible high prevalence values. However, this distance may be an underestimation due to the parameter values being taken from related host and pathogen species being an underestimation, therefore it is likely that a radius of 500 m will encapsulate the transmission distances of VHSV. Likewise, a more cautious estimate could have been obtained, should this VHSV strain be more conserved than ISAV or other VHSV strains, however, it is likely that the offshore residual current speed are a significant overestimation for the inshore areas where salmon farming occurs in this region. Previous work has demonstrated that for a range of pathogen species, transmission distances diminish with reduced residual current speeds[31].

Although the distances may appear low in comparison to the radius established for disease management areas for ISA control, it must be noted that salmon biomass on farms is between 10 - 20 times greater than the biomass of wrasse held on the positive sites identified in Shetland. Although, it is not a linear

[56] Murray A.G., Munro L.A., Wallace I.S., Berx B., Pendrey D., Fraser D. and Raynard R.S. 2010. Epidemiological investigation into the re-emergence and control of an outbreak of infectious salmon anaemia in the Shetland Islands, Scotland. *Diseases of Aquatic Organisms*, **91**, 189–200.

relationship between biomass and potential transmission distances, the general pattern is that the lower the biomass held on the pathogen positive farm, the lower the transmission distances of shed pathogen[31].

Appendix 2

Wild Fish Sampling Protocol

I.S. Wallace

Sampling Strategy

This uses the approximate proportions of the main species present in the catch to determine numbers to sample. This basis for choosing this strategy is that VHSV appears to be ubiquitous in species of wild marine fish therefore targeting specific species may result in under detection in the local populations.

Sampling Location

Sampling will be undertaken in a laboratory at the NAFC Marine Centre with the fish being delivered to the back door each day.

Target Tissues

Kidney, spleen, heart and brain will be taken from each fish into VTM.

Pool Size

Fish will be pooled in tens.

Tissue Sampling Protocol

- ► Sort fish into respective species outside and count the number of each (roughly)
- ► Based on proportions of each species present decide on the number of each to sample
- ► Bring the fish inside
- ► Wearing sterile disposable gloves, lay 10 (depending on fish size) fish on a stainless steel dissecting tray overlaid with paper towel. Place round fish with head to the left and flat fish eyes up (will depend on if left or right eyed)
- ► Complete the sample record sheet

- ► Using 70% ethanol solution and paper towel surface sterilize each fish
- ► Using a sterile disposable scalpel cut open the body and cranial cavities of each fish in the pool (scalpel 1)
- ► Change gloves and scalpel (scalpel 2)
- ► Open the body cavity of the first fish using a gloved hand, remove samples of spleen, kidney and heart tissue and place in a labelled VTM tube (scalpel 2)
- ► Repeat for each fish in the pool
- ► Using the same scalpel (scalpel 2) remove a sample of brain tissue and place in the same labelled VTM tube
- ► Repeat for each fish in the pool
- ► **Note:** Final tissue volumes in each pool must be between the minimum and maximum marks on the virus reference tubes
- ► Used scalpels and forceps go into a sharps container
- ► All sampled fish are to be double bagged in heavy duty black bin liners
- ► Used towel roll and gloves go into a separate black bin liner
- ► Clean the tray with a solution of 70% ethanol
- ► The labelled VTM tube goes into a stainless steel rack contained within a polystyrene cool box and freezer blocks
- ► When the bags are full, or at the end of the sampling day, they must be put into the freezer adjacent to the laboratory and labelled as to the contents.

Additional

- As the site adjacent to the sampling laboratory has a movement order for VHS in place careful bio-security must be implemented at all times.
- On arrival at the laboratory, prior to any sampling taking place, all sampling surfaces within the sampling laboratory are to be disinfected using a solution of virkon.
- Warning signs will be displayed on the outside of the sampling laboratory doors to inform non-sampling personnel of the nature of the work being undertaken and to stay out.
- Bring into the laboratory only what is necessary to undertake the sampling. Store un-used consumables within the vehicles until required.
- On completion of sampling all bench surfaces and the floor are to be cleaned and disinfected using a solution of virkon.

- Any equipment being returned to the MSS laboratory must be cleaned and disinfected prior to leaving the site and again on return to the MSS laboratory comprising: stainless steel dissecting trays; ethanol spray bottles; polythene boxes used to transport samples and any personal protective equipment such as wellington boots and oil skins. Used sharps containers must be locked shut and tied in clear polythene bags for incineration.
- Waste bags containing sampled fish and used consumables are to be incinerated at the local Shetland Islands Council energy recovery plant.

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