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Report into the Epidemiology and Control of an Outbreak of Infectious Salmon Anaemia in the Shetland Islands, Scotland

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Summary:

An outbreak of infectious salmon anaemia (ISA) in the Scottish Shetland Islands during 2008/9 is described during which six sites were confirmed ISAV positive. Spread of the virus via movement of fish between marine sites, harvest vessels, movements of smolts and wild fish appear to have been of little or no importance. The spread is associated with hydrodynamic currents, although local intra-company activity may have caused some spread. The application of a statutory control strategy by Marine Scotland, based on the use of its established model (Anon 2000) has apparently limited the occurrence and economic impact of ISA to management area 3a; however spread within this area has been extensive. This localised water-borne spread is in contradiction to a previous outbreak in 1998/9 which was spread over a wide geographic area by transport of fish and harvest vessels. The development of industry codes of practice and good biosecurity procedures, following the 1998/9 outbreak, that limited marine site-to-site movement of live fish and improved disinfection of vessels and processing plant waste, may explain why the 2008/9 spread of infection was localised. Depopulation of confirmed sites is a key element of eradication and this was achieved within 7 weeks of confirmation, although the last confirmed case suggests subclinical infection may persist undetected for months. The potential sources of ISAV infection that were investigated did not determine the origin of the 2008/9 outbreak. Local evolution from an avirulent strain of ISAV; importation of ova; or association with movement of equipment could have caused the outbreak. The virus responsible for the 2008/9 outbreak belongs to a different genogroup (group 1) to the 1998/9 virus (group 3). The intensive cultivation of salmon farming, close proximity of sites and historic absence of synchronous fallowing of management areas is considered to have increased the risk of disease outbreaks and their re-emergence, such as ISA in the Shetland Islands. A policy of synchronous fallowing and stocking of sites within management area 3a is being considered by industry in consultation with Marine Scotland to address this risk. Where movement of fish occurs between sites in

different management areas, this represents the greatest risk of regional-scale spread of diseases such as ISA. Controls appear to have been effective in minimising that risk.

1. Introduction

Infectious salmon anaemia (ISA) is a highly infectious orthomyxoviral disease of farmed Atlantic salmon (*Salmo salar*) caused by ISA virus (ISAV) which is notifiable to the World Organisation for Animal Health (OIE 2009). Clinical ISA has caused major losses to salmon farmers in six countries (OIE 2009). The disease was first reported from Norway in 1984 (Thorud and Djupvik 1988), where it is still widespread (Lyngstad et al. 2008). Subsequently it occurred in Canada, the USA, the Faeroe Islands and Chile (Mardones et al. 2009). None of these 5 territories have officially eradicated ISA (Mardones et al. 2009). There are no recent reports of ISA from the Faeroes, but vaccination is practiced and vaccinated salmon may be carriers of the virus (OIE 2009).

An outbreak occurred in Scotland in 1998/9 (Anon 2000; Stagg et al. 2001; Stagg 2003) and this was eradicated at a cost then of over £20M (Hastings et al. 1999). During this outbreak, ISAV was confirmed on 11 sites with an additional 34 suspect sites being identified which were scattered across virtually the entire region of Scottish salmon farming before its eventual eradication (Stagg 2003). At the time of the outbreak, native Scottish wild salmonids were screened for ISAV and evidence of infection, but not ISA disease, was found in Atlantic salmon, brown and sea trout (both *Salmo trutta*) (Raynard et al. 2001).

The ISA virus can infect salmonid species such as brown trout, sea trout and rainbow trout (*Oncorhynchus mykiss*), which can become carriers but do not suffer clinical disease (Nylund and Jakobsen 1995). Both the freshwater brown and anadromous sea trout are native to Scotland and although rainbow trout are non native they are sometimes found in the wild due to stocking for angling purposes or as fish farm escapes. The virus has also been reported to be replicated in non-salmonid species such as herring (*Clupea harengus*) (Nylund et al. 2002) and Atlantic cod (*Gadus morhua*) (Grove et al. 2007) although these reports relate to experimental challenges rather than to wild infections.

Infectious salmon anaemia does not represent a risk to human health since the virus does not grow at temperatures above 25 °C and is inactivated at 37 °C and therefore unable to survive at human body temperatures (SANCO/C3/AH/R18/2000).

This report describes a second outbreak of ISA in Scotland that occurred during 2008/9. This has been geographically confined to a relatively small area in the southwest of the Shetland Islands, (Anon 2009a). Six infected sites were confirmed and depopulated; the management area (MA) then underwent a synchronised fallowing. However, controls on the MA remain in place until a 2 year programme of surveillance and testing has been completed to allow the Shetland Islands MA 3a to be re-declared as part of the UK ISA free zone. Scottish Government policy is to confine and eradicate ISA, in accordance with EU Council Directive 2006/88/EC, and to take appropriate measures to regain and maintain its disease-free status.

2. Background

2.1. Farm Distribution of ISA Susceptible Fish

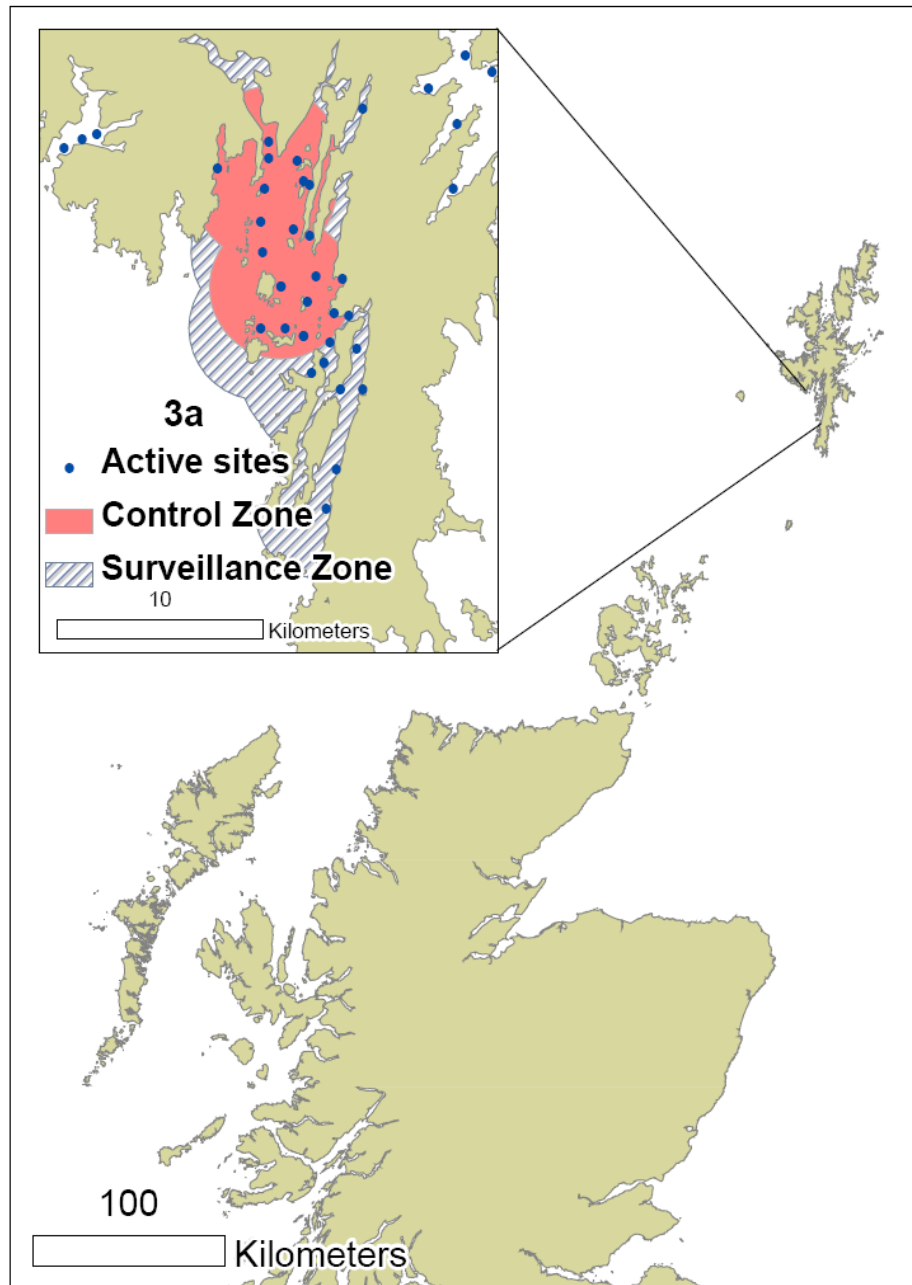


Figure 1 A map of Management Area 3a (MA 3a) inserted into a map of Scotland. The entire MA is in the surveillance zone, while the control zones were based on sites within a tidal excursion distance of confirmed or suspect sites. The initial control zone was placed around site A, the origin of infection, followed by two suspect sites, and expanded as further cases were confirmed.

In 2008 Scotland produced 128,606 tonnes of farmed Atlantic salmon from 257 marine sites and 2628 tonnes of sea-farmed rainbow trout from 9 sites (Walker 2009). A further 311 tonnes of brown and sea trout were also produced but separate statistics are not published for marine and freshwater farms. A third of Scottish salmon production, 42,593 tonnes, came from the Shetland Islands (Walker 2009) in the far north of Scotland (60 °N) (Figure 1).

Scottish salmon production is divided for the purposes of disease control, including ISA, into MAs (Anon 2009b), which are defined on the basis of overlapping tidal excursion zones. These MAs use circles with radii of 3.6 km in Shetland (7.2 km elsewhere in Scotland) based partly on a simple but robust tidal model (Stagg 2003). A MA is defined by all sites with overlapping radii and stretches until a break occurs that is large enough that the circles do not overlap (i.e. a separation distance of $> 2 \times 3.6$ km). These MAs were originally devised as part of the controls for the 1998/9 ISA outbreak (Stagg 2003) and simple modelling indicates they are likely to be appropriate for ISA control, if not for other pathogens (Murray et al. 2005).

In 2008/9 ISA cases were restricted to one MA in south west Shetland (MA 3a) (Figure 1). This MA contained: 42 registered finfish farm sites on which controls were set; 16 inactive sites; 11 fallow prior to the outbreak; 13 sites which were populated with salmonids at the time of the outbreak (having not yet been harvested), and 2 sites producing non-susceptible species ([2006/88/EC Part II, Annex IV](#)), one containing halibut (*Hippoglossus hippoglossus*) and the other, a small research site, holding both ballan and goldsinny wrasse (*Labrus bergylta* and *Ctenolabrus rupestris*). The MA is the largest in Scotland (Anon 2009b) and produced over 10% of Scotland's farmed salmon in 2007. The MA also contains a fish processing plant situated at the port of Scalloway.

2.2. Detection of ISAV

2.2.1. Surveillance

Fish Health Inspectors (FHIs) from the Marine Scotland Marine Laboratory carry out routine disease surveillance at fish farms in accordance with current legislation, including EU Council Directive 2006/88/EC and The Aquatic Animal Health (Scotland) Regulations 2009 and in accordance with the Fish Health Inspector's charter. However, the putative index case of the 2008/9 outbreak (i.e. the site at which the pathogen was first found) was detected as a result of intelligence-led surveillance when the site, amongst others, were inspected following reports from the Shetland mainland of health problems, large scale mortality and disposal of dead fish. These losses were attributed to sea lice (*Lepeophtheirus salmonis*) by industry and subjected to further investigation by the FHI. As part of these investigations, an inspection at site A was undertaken on 9th December 2008, when FHIs took diagnostic samples from 5 moribund fish and on obtaining results indicating ISAV

infection, returned on 16th December to take a further statutory sample of 150 fish. Following initial investigations and site inspections, at that point in time, this site was the only site at which positive test results for ISAV were obtained.

Following confirmation of ISA on this site on 2nd January 2009, MA 3a became a surveillance zone, and within this all sites within 3.6 km of confirmed or suspect sites became a control zone (Figure 1). Marine Scotland FHIs visited sites within MA 3a on a monthly basis and collected records of site mortality levels. Initially 150 fish samples were taken at all sites within the control zone thereafter monthly inspections were conducted, while bi-monthly inspections were undertaken at all sites within the outlying surveillance zone. If clinical, and/or gross signs of disease were evident the sites were subject to 5 to 10 fish full diagnostic sampling. If results indicated possible infection and suspicion of disease on site, but test results did not confirm ISA, a further 150 fish sample was collected. Confirmation was possible from the diagnostic sample when results were obtained in accordance with EU directives. Sites in other MAs that had possible past contacts with MA 3a, including sites from which fish had been moved to or from MA 3a and a site near a processing plant (fish obtained from MA 3a had been held in a cage at the plant prior to processing), were also subjected to 150 fish tests and bimonthly inspections. Where direct contact was made with control zone sites, these sites were subjected to a 150 fish test.

Sites that receive fish from ISA confirmed sites (before these were confirmed and further movement therefore banned) are automatically classified as officially suspect.

Passive surveillance methods (where the official service follows up reports of possible disease e.g. from farmers or vets (Hadorn et al. 2008)) were used to enhance surveillance when one site was re-visited ahead of schedule after reports by a veterinarian of possible ISA and elevated mortalities. As ISA is a notifiable disease there is a legal obligation on anyone with reason to suspect its presence on a fish farm to report this suspicion to the competent authority (FHI) (The Aquatic Animal Health (Scotland) Regulations 2009).

2.2.2 Diagnosis of ISA

Diagnosis of ISA was carried out in accordance with EU legislation, EC/91/67 followed by EC/2006/88, as implemented by current regulation; following European Law, Commission Decision 2003/466/EC, and following the OIE (2009). A health inspection is a visit by an official service for the purpose of conducting health checks on a farm or zone. This involved: clinical observation, by qualified and experienced fish health inspectors, of gross pathology associated with fish disease; prompting a diagnostic investigation which includes approved laboratory diagnostic tests as provided for in regulation, involving light microscopy; quantitative Real-time polymerase chain reaction (qPCR) and virus isolation processed by the diagnostic

laboratories on samples taken by FHI. Additional diagnostic methods were used, where appropriate, as approved by the OIE and EU law (Table 1).

Diagnostic methods are detailed in Appendix 1, and summarised in the following text. A positive qPCR result (Snow et al. 2006; 2009) in the 5-10 fish diagnostic sample does not automatically result in ISA confirmation, or even official suspicion, nevertheless this triggers the collection of a further 150 fish sample. If gross pathological signs of ISA, such as dark liver, are present in combination with qPCR positive results then the site will be placed under official suspicion, likewise qPCR results can be confirmed by indirect tissue imprint fluorescent antibody test (IFAT) (Stagg et al. 1999) or histology. Virus isolation, with virus identified using TO cell susceptibility (Wergeland and Jakobsen 2001) plus IFAT (Falk et al. 1998), alone is enough to result in the confirmation of ISA even if unsupported by other diagnostic tests, provided there are at least two independent isolations. Confirmation was made following a case review by senior diagnosticians, the leader of the FHI and the programme director.

Table 1

Evidence use to provide official confirmation of ISA

Test 1	Test 2	Result
Mortality, clinical signs and pathology (e.g. microscopy) consistent with ISA	Virus isolation	Confirmed
	qPCR	Confirmed
	IFAT	Confirmed
Virus isolation from 2 independent samples		Confirmed
Virus isolation	qPCR	Confirmed
	IFAT	Confirmed

Diagnostic tissue samples obtained from farmed fish for qPCR and virus isolation were pooled using tissue from 5 individuals to optimise diagnostic costs. Diagnostic samples may consist of a few moribund individuals and in such cases smaller pools or individual fish were tested. As part of the surveillance wild fish were also sampled (Wallace et al. 2009), freshwater fish were not pooled owing to the smaller numbers available while marine fish were pooled but sometimes pools of <5 fish were used because the numbers of the different species caught were not always divisible by 5; fish of different species were not pooled together.

Using methods described by N  rette et al. (2008), virus isolation was found to have a specificity (Sp) of 100% for ISA but is slower, taking up to 4 weeks, and has less sensitivity (Se) than qPCR; isolation Se = 88.7% as opposed to 93% for qPCR (N  rette et al. 2008). The qPCR was found to be only 99% specific, which means significant numbers of false positive results are possible when large numbers of fish are sampled. For this reason unsupported qPCRs are treated as false positives if they could not be confirmed by other indicators of ISA. Positive qPCR results can be

generated by detection of the putatively avirulent HPR0 strains of ISAV (McBeath et al. 2009) or possibly even by genetic material from totally unrelated organisms (Gregory et al. 2007), although in this case a different Real Time-PCR test was used. These values of Se and Sp may differ slightly in Scotland as diagnostic methods and the viruses are slightly different. However, differences are likely to be small and a set of samples have specifically been collected (data not discussed here) that will allow future estimates to be made for Scotland using the same analysis as N  rette et al. (2008).

During the laboratory analysis the qPCR diagnostic tests are confirmed using three replicate reactions taken from one homogenised sample of material. Only if all three replicates are positive is the test positive. This replication increases Sp with respect to errors, but does not correct for a systematic false positive, such as presence of HPR0. The requirement of replication could potentially reduce diagnostic sensitivity when viral titre (concentration in the tissue) is close to the theoretical limit of detection by qPCR.

The examination of stained tissue sections provides unique information regarding tissue changes that will include ISA as well as any other infectious or non-infectious co-infection. For this sections from the gut; heart; spleen; kidney; liver; pancreas and skeletal muscle were stained with haematoxylin and eosin and examined by light microscopy.

The ISAV responsible for the outbreak, 2008-0982, was isolated and its haemagglutinin-esterase protein gene sequenced. This has been compared with the sequence from other EU-G1 isolates, including the 1998-390 virus that was isolated in the previous Scottish outbreak in 1998/9.

2.2.3 Response to Confirmation of ISA

On suspicion of ISA, a thirty day notice was placed on the suspect site A, 12 December 2008, thereby imposing movement restrictions for this period. On confirmation of ISA at the index site the Scottish Government imposed restrictions preventing movement into and out of MA 3a, which formed control and outlying surveillance zones (Anon. 2009b) and Figure 1). Within the control zone (3.6 km from a suspect or confirmed site) inspection was increased with monthly visits and reports of mortality from sites. The area within 3.6 km of the index site, and subsequently of other suspect or confirmed sites, formed the control zone, which thus expanded as further sites were confirmed (Figure 1). Scottish Government policy is for rapid removal of all fish from ISA confirmed sites in accordance with EU Council Directive 2006/88/EC. Fish of harvestable size, not displaying external signs of clinical ISA disease, as observed individually at the point of humane killing of the fish, were taken for processing at an approved biosecure plant. Fish that were undersized or displaying gross disease signs were discarded and disposed of as

waste material, by an approved manner, under current waste disposal legislation. Historically in Scotland (during the previous ISA outbreak in 1998-99), confirmed sites were fallowed for 6 months while those in the control zone fallowed synchronously for 3 months (Stagg 2003). All sites in the surveillance zone were fallowed for 6 weeks but synchronisation was not achieved. As the fallowing period in 2009-10 was synchronous within the control zone, it started when the last site in the area has been cleared of fish. Further details, including notification procedures are provided in Appendix 3.

2.3. Investigating the Potential Routes of Spread

The hazards associated with the risk of spread of infection were investigated. Risk assessment, results and analysis are described in section 3.3.

2.3.1. Movements of Fish and Harvest Vessels

Marine Scotland Science provides advice to Marine Scotland Policy on the movements of fish between sites under control and also collects data on such movements (Munro and Gregory 2009). Live fish movements are recognised to be among the most effective ways of spreading pathogens (Murray and Peeler 2005) and therefore these records have been analysed to determine if the movements of fish between sites within MA 3a and between sites in this MA and other MAs contributed to spread of ISAV.

Vessels that deliver fish to processing plants can spread infection, including ISAV, when they return from the processing plant (Murray et al. 2002; Munro et al. 2003). Two processing plants were used to process fish produced at sites within MA 3a, one at Scalloway, Scalloway Processing Plant (SPP) within MA 3a, and the other in Lerwick, Lerwick Processing Plant (LPP) on the east coast of Shetland (Figure 7). The FHIs obtained records from the logs of vessels involved in transporting fish between sites in MA 3a and these processing plants. They also visited the processing plants to assess biosecurity and disinfection regimes, as it would require a failure of biosecurity to contaminate visiting vessels causing them to become vectors.

2.3.2. Vertical Transmission

There is currently scientific disagreement on the occurrence of vertical transmission of ISAV (Lyngstad et al. 2008; Vike et al. 2009). Historically ISAV was not considered to be vertically transmitted but in light of the current debate which does not rule out the option of vertical transmission, it is important to consider its potential effects. If vertical transmission did occur then this could be significant both as a potential source of the Shetland outbreak, since imported ova are used, and as a potential route of spread within Scotland and beyond via broodstock produced in

Shetland. It is also possible that ISAV could be horizontally transmitted e.g. on the outside of poorly disinfected eggs (pseudovertical transmission). We therefore assess sources of input of smolts to Shetland and conduct a risk assessment on the potential exposure of ova produced from broodstock within Shetland.

2.3.3. Wild Fish

It is possible that ISAV may be transmitted by movements of wild fish (Nylund and Jackobsen 1995; Raynard et al. 2001). During this outbreak two wild fish surveys were undertaken by Marine Scotland (Wallace et al. 2009), one of wild marine fish from within MA 3a, the second of fish from fresh waters draining into the MA (Figure 2).

The wild marine fish survey was conducted at 3 locations (Figure 2) and consisted of multiple trawls to gain good numbers of fish for screening. These trawls were carried out between the 9 and 11 February 2009. Samples were pooled in groups of 5 fish, but organs were collected individually (kidney, gill, brain and heart), e.g. a pool of heart material from 5 fish.

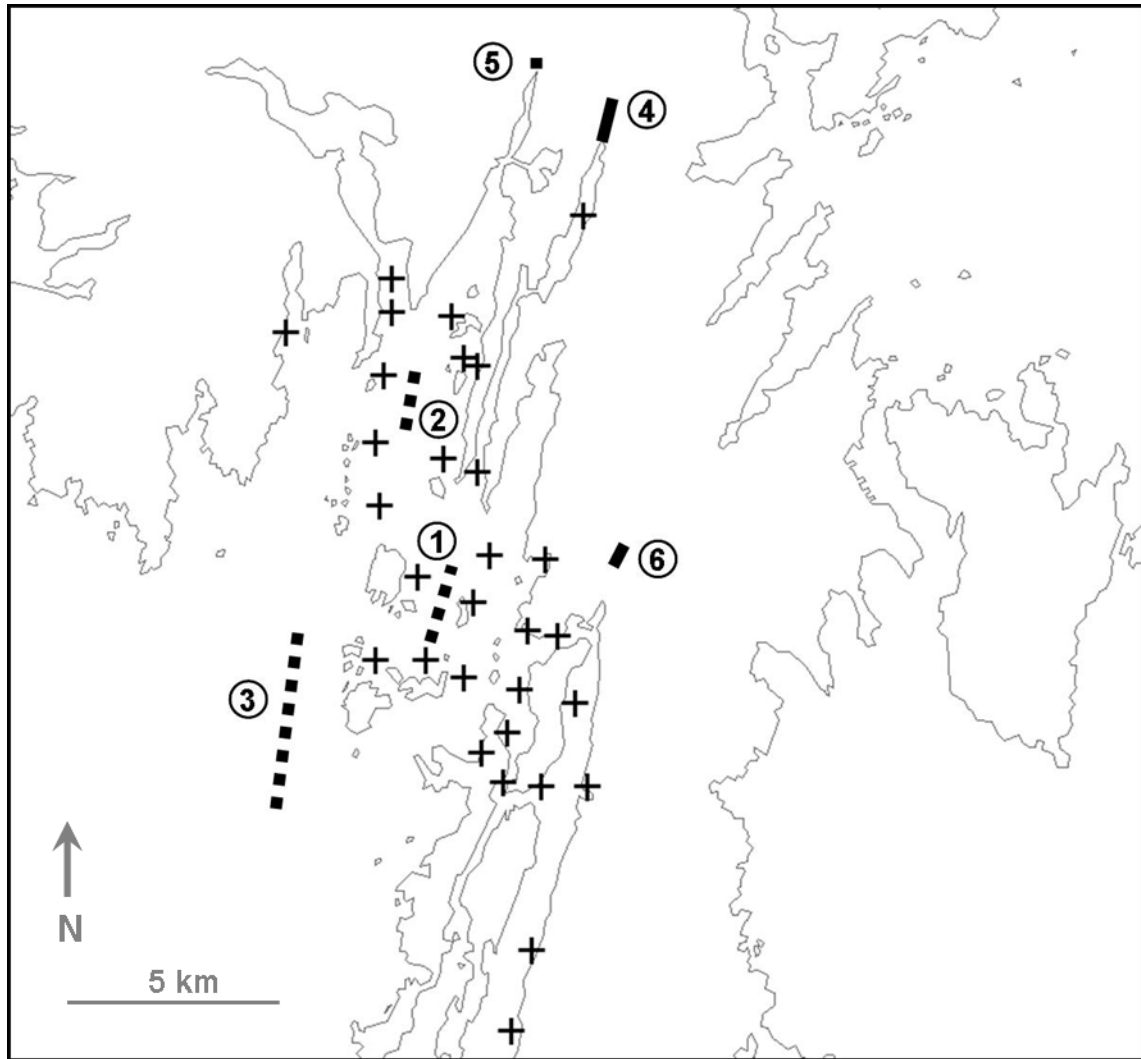


Figure 2 Wild fish sampling locations for the ISA investigation: + = fish farms in MA 3(a); dotted lines are numbered marine tows; solid lines are numbered electrofishing sites and numbers refer to the catch breakdown tables.

The freshwater fish were sampled from 3 burns (1 sampled over 2 days) on the 23 – 25 March. The slightly later date of sampling was chosen to allow the freshwater temperatures to increase making the fish more active and easier to catch (personal experience). Additionally this would increase the chances of catching migratory sea trout returning to freshwater at this time of year. Fish were caught using electrofishing and the fish were sampled individually, with the same organs collected as for wild marine fish.

In addition, 2 saithe (*Pollachius virens*) found in a cage on an ISA infected salmon farm were opportunistically sampled taking the same tissues as listed above.

2.3.4. Hydrodynamic Spread

The ISAV is transmitted via water even from fish that are not displaying clinical disease signs (Totland et al. 1996). However, shedding increases 2 days before death (Gregory et al. 2009) so populations with a high prevalence of clinically infected fish represent a high risk of pathogen spread.

Several studies have shown that sites in proximity to infected sites are more likely to become infected. Jarp and Karlsen (1997) identified an enhanced risk of infection if infected farms (odds ratio (OR) 8, Kelsey et al. 1996) or slaughterhouses without disinfection (OR 14.6) were within 5 km of sites, with the odds increasing as the distance decreased. Modelling by Scheel et al. (2007) found infected sites within 5 km to be of comparable risk to sites with shared ownership. Lyngstad et al. (2008) found that infected sites within 10 km were likely to share ISA viruses of the same genogroup indicating possible shared infection. Aldrin et al. (2010) showed risk of infection increased rapidly with inverse distance from the nearest infected neighbour, provided this was <10 km away. Using currents estimated from a hydrographic model Gustafson et al. (2007) observed that sites down current from an infected site were more likely to pick up infection than those situated up current.

The spatial pattern of the outbreak was assessed to consider possible spread of pathogen amongst sites, caused by water movements. It is important to consider the role of infected sites within a tidal excursion of adjacent uninfected sites and the risks of transmission. Within management area 3A there was a high concentration of farm sites within close proximity to each other, enhancing the potential risk of hydrographic and biological interaction amongst them.

3. Results and Discussion

3.1. Chronology of the Outbreak

At the time that the putative index case was confirmed (2 January 2009) there were 12 stocked Atlantic salmon farms one trout farm and two non-susceptible species farms in MA 3a. The site testing history is summarised (Table 2); only qPCR and virology results from the last diagnostic test taken from each site are listed. Other results available for some cases but not listed here included immunohistochemistry (IHC); IFAT; histology and clinical disease signs. A complete list of diagnostic test results from samples taken from diagnostic, statutory or research purposes is included in Appendix 2. Due to the long time period required to obtain a virology result, sites D, E, and F were confirmed by other positive diagnostic tests as described in the following text. When the virology results were obtained, from earlier diagnostic tests or statutory 150 fish tests, these positive results supported confirmation. Tests that produced negative diagnostic results may have occurred earlier than the results listed in Table 2. One sample (from site F) generated a positive qPCR that was not confirmed as there were no clinical or gross signs of ISA and no other positive diagnostic test results, until 10 months later when the site did test positive during harvesting.

The putative index case site A was visited on 9 December 2008 as part of an investigation into fish health problems ascribed to sea lice within MA 3a. Diagnostic sampling resulted in a positive ISAV qPCR so the site was re-visited and 150 fish were sampled in 30 pools. The results of testing confirmed the presence of ISAV by qPCR (8/30) and virology (7/30) on the 2 January 2009. Harvesting was ongoing and the site was followed by 20 December (Figure 3.I). However, at the management area level, movement restrictions and the previously outlined enhanced surveillance regime were implemented.

Table 2

Chronology of the ISA outbreak illustrating the date of a positive sample or last negative sample before harvest. Only qPCR and virology results (in pools of 5 fish) are listed, other results by IHC, IFAT, and histology and diagnostic signs were also available for some cases. Sites harvested before testing began are not listed in table. The number of fish on site is that at the end of 2008, except site A for which this is the number before harvesting began.

Site	Owner	Species	Last Date Sampled	Date Confirmed	Test Result	Date Depopulated	Fish on site
A	C1	Salmon	16/12/2008	02/01/2009	PCR 8/30 V 7/30	20/12/2008	87,000*
B	C2	Salmon	12/01/2009	30/01/2009	PCR 4/30 V8/30	06/03/2009	316,000
C	C2	Salmon	18/03/2009	20/03/2009	PCR 16/30 V15/30	08/04/2009	263,000
D	C2	Salmon	14/05/2009	18/05/2009	PCR 1/1 V1/1	02/07/2009	284,000
E	C3	Salmon	19/05/2009	21/05/2009	PCR 25/30 V26/30	27/05/2009	6,000
F	C4	Salmon	13/01/2009	30/10/2009	PCR 1/1 V 1/1	26/11/2009	286,000
g	C1	Salmon	06/01/2009	N/A	PCR 0/30 V 0/30	07/01/2009	297,000
h	C1	Salmon	08/01/2009	N/A	PCR 0/2	14/01/2009	178,000
i	C2	Salmon	14/01/2009	N/A	PCR 0/30	16/01/2009	301,000
j	C1	Salmon	17/01/2009	N/A	PCR 0/30	20/01/2009	249,000
k	C4	Salmon	16/01/2009	N/A	PCR 0/30	28/01/2009	60,000
l	C2	Salmon	13/01/2009	N/A	PCR 0/30	25/02/2009	250,000
m	C5	Trout	29/01/2009	N/A	PCR 0/30	02/04/2009	142,000
n	C6	Halibut	N/A	N/A	N/A	27/04/2009	6,000
o	C3	Wrasse	N/A	N/A	N/A	N/A	380

The movement restrictions imposed when the index case was confirmed prevented any movement of fish into or out of the surveillance zone, although movement of live fish in sealed well boats for harvesting at LPP were authorised following a risk assessment, ensuring good biosecure practices (see later).

Two sites had received fish from the putative index case (site A) and for this reason were automatically placed under official ISA suspicion. One of these sites had already been harvested and the other (site g) was subject to a 150 fish test which was negative by both qPCR and virology. The last transport of live fish from A to g had occurred on 27 June 2008 approximately six months before ISA was detected at the index site A. This strongly suggests ISA was absent or, if present, at a very low level on site A in June, i.e. ISA emerged within the last six months of 2008. While it is possible that another site was the undetected true index case, the observations

strongly suggest ISA had emerged recently, which has important implications for the source and potential spread of the outbreak.

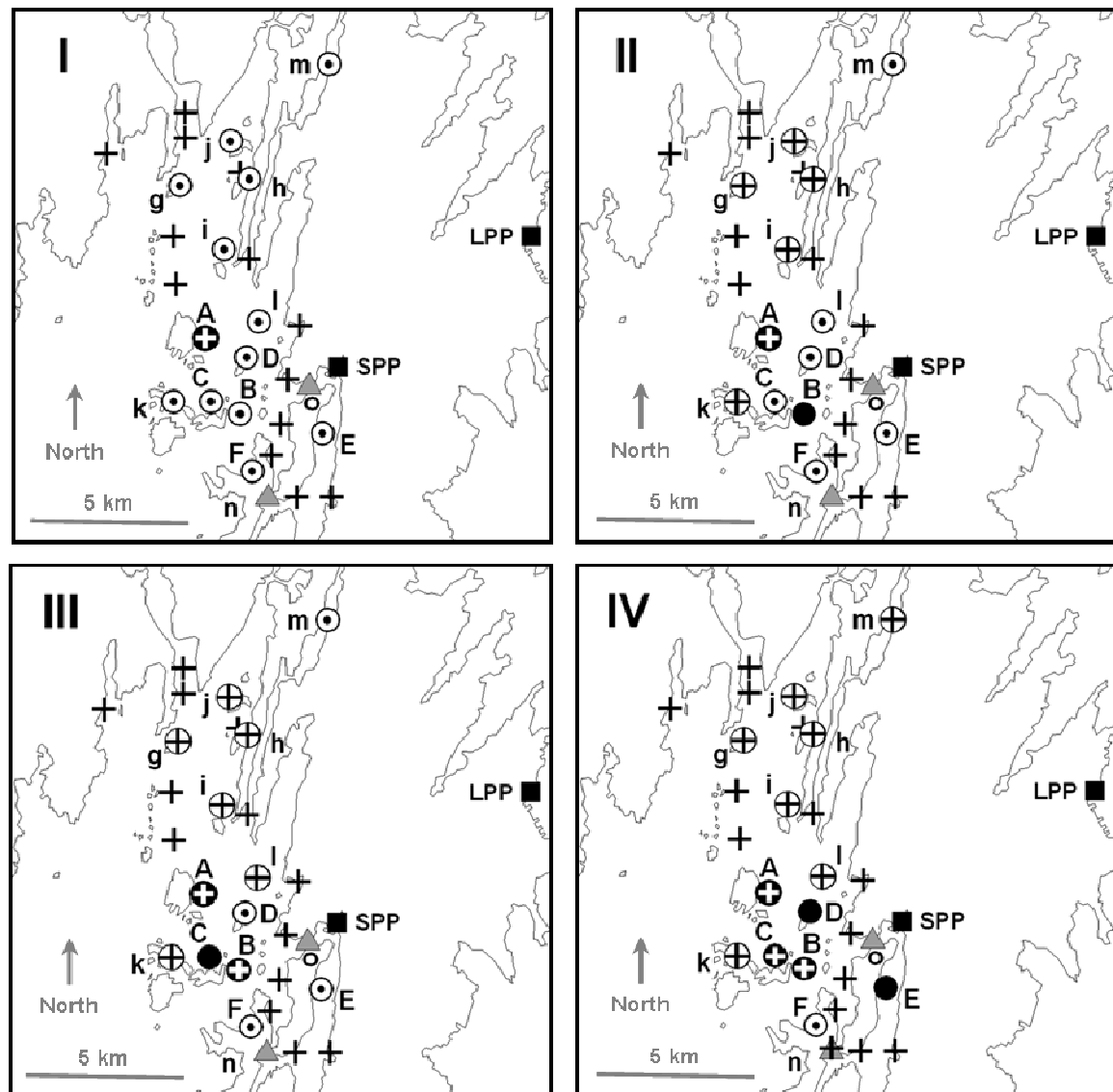


Figure 3 Maps of MA 3a on: (I) 31st December 2008; (II) 31st January 2009; (III) 31st March 2009 and (IV) 25th May 2009. The letters refer to the farms listed in Table 2: + = farms depopulated prior to the outbreak; ⊙ = uninfected farms; ⊕ = farms fallowed during the outbreak; ● = ISA positive farms; ⊕+ = ISA positive farms fallowed; ▲ = populated farms containing non-susceptible species (halibut, ballan and goldsinny wrasse); ⊕▲ = fallowed non-susceptible species farm (halibut) & ■ = processing plant. (Two fallow farms included in Figure 2 are located off the foot of this map).

Five sites (g-k) were harvested during January 2009 including the officially suspect site, g, which had by then been confirmed to be ISAV negative after a 150 fish test. All of these sites had tested negative, most at the 150 fish level, although site h was only subject to a smaller diagnostic test, prior to it being fallowed. Thus half of the

salmon sites in MA 3a that had been populated in December 2008 were empty by the end of January 2009 (Figure 3.II), as a result of normal planned harvesting activity.

There were diagnostic indications of ISAV from two sites in January 2009, however, these were not confirmed. One site (D) tested positive in 2 of 3 replicates from one pool when tested by qPCR. This, as described in the methods, was officially a negative result however it might indicate exposure to infection. Five months later on 14 May 2009 positive tests were obtained from this site which became the fourth to be confirmed. Site F tested positive by qPCR on the 7 January 2009, however, it was not confirmed by virology. A subsequent 150 fish test from this site on 13 January was negative by both qPCR and virology. However, the site was confirmed as ISAV positive nearly 9 months later on 30 October 2009 during harvesting. Even if these sites had been positive, due to them being located in MA 3a, they were in any case under movement restrictions and enhanced surveillance. As such, their treatment as negative in the absence of clinical ISA was considered to be of low risk, although their subsequent histories suggest sites D and F may have been carrying infection for a long time before they were confirmed ISAV positive.

During January 2009 site B tested positive by qPCR (4/30) and virology (8/30) to become the second confirmed case of ISA on 30th January 2009. This site was cleared of fish by the 6 March. Another ISAV negative site, I, was harvested on 25 February. The trout producing site m was culled on 2 April for welfare reasons, although the fish tested ISA negative. The site was located in shallow water so as water temperatures increased and the fish grew larger their stress was increased. Traditionally these trout were moved to a site in a different MA but this was prohibited by ISA movement restrictions. By 7 March 2009, within MA 3a, there were 7 stocked sites: 4 salmon farms; the sea trout site and the 2 non-susceptible species sites (Figure 3.III).

Site C, adjacent to positive site B, tested positive from diagnostic samples taken on 11th March 2009. This became the third confirmed ISA positive site following a 150 fish test on the 20th March (16/30 by qPCR and 15/30 by virology). The site was fallow by the 8th April. In April, the halibut site n was harvested, but these fish are non-susceptible so this is of no relevance to ISA control (apart from increased boat traffic).

A fourth site D was confirmed ISA positive on the 18 May 2009 from samples collected on the 14 May. This site was rapidly confirmed on the basis of not only qPCR (1/1) and IFAT (1/1) testing but also histological signs of ISA and increased mortality. However, this site was slow to fallow and was cleared of fish by 2 July.

A fifth site E was confirmed at nearly the same time (21 May) on the basis of samples taken on 19 May (25/30 qPCR; 26/30 virology and 12/12 by histology). The site had

been sampled after a veterinarian reported signs consistent with ISA. This was a small research site and was followed by 27 May (Figure 3.IV).

By the second week of July 2009 only site F remained stocked with susceptible fish in MA 3a as there was insufficient evidence, from monthly inspection at that time to confirm the presence of the disease. This site was close to sites from which infection had been reported in spite of an early positive qPCR result (7 January 2009), signs of ISA were not observed until October 2009. Visiting FHI's observed gross pathology, prompting the sampling of moribund fish, leading to confirmation of ISAV. The site was confirmed positive on 30 October on the basis of cell culture, qPCR and clinical signs. As no other sites containing susceptible species were populated for 4 months prior to this it is likely infection persisted undetected on F for at least this time period.

Site o was a very small (Table 2) tank-based research site containing non-susceptible species. This was not considered to be a risk for ISA and so was not depopulated.

3.1.1. Diagnostic Testing Outside MA 3a

All the sites located out with MA 3a that were tested gave negative diagnostic results for ISAV. The complete list of diagnostic test results from within Shetland but outside MA 3a are presented in Table 2 of Appendix 2. Most fish were tested by qPCR and virology, but some by qPCR only, 1352 samples were taken from 685 fish. A total of 15 sites were sampled by qPCR and 12 of these were also tested by virus isolation. Sampling was concentrated on high risk sites with 600 fish taken from 4 sites that might have been exposed directly or indirectly by fish movement: two in MA 4a, one in MA 3b and one in MA 2b, this last being the site nearest to LPP (Figure 6).

3.1.2. Clinical ISA

The positive sites were confirmed on the basis of qPCR and virology testing, although pathology allowed a more rapid confirmation of sites, including site D, before the virology results (which later supported the confirmation) were available. During the outbreak, evidence of both infection and clinical disease were found in MA 3a. Pathological changes associated with ISA; observations from fish sampled at each confirmed site are illustrated in (Table 3). Gross pathology is determined by OIE guidelines and a clinical score sheet used to assess liver colour score.

Experimentally challenged salmon using virus obtained from site B developed diagnostic features of clinical ISA and suffered elevated mortality, although mortality was slower to occur than from the 1998-390 strain (K. Urquhart in prep.). The 2008-0982 ISAV was undoubtedly the causative agent of disease.

Table 3

Diagnostic features associated with clinical ISA found in fish from confirmed sites A-F. Liver colour is on a scale where values ≥ 8 raise suspicion of ISA.

Site	Pale Gills	Zoned Gills	Pale Heart	Dark Liver	Bloody Ascites
A		Y			Y
B		Y	Y	10	Y
C		Y	Y	9	Y
D	Y		Y	8/9	
E	Y	Y	Y	9/10	Y
F	Y	Y		8/9	Y

The confirmed sites C, D and E all exhibited mortality rates above 1% week⁻¹ over several weeks in the worst affected cages. Mortality at sites B and F was generally low only reaching 1% week⁻¹ on one occasion at each site. Mortalities $>0.05\%$ week⁻¹ were widespread across most cages on affected sites, except F. At confirmation, site A was fallow, however mortality rate was reportedly as high as 7% per week, on the worst affected cage at the time of the index case. Elevated mortality was attributed by the site operators to causes other than ISA and certainly other pathogens were reported as present and confirmed by laboratory diagnosticians. The fish on all sites exhibited gross pathology, lethargy and morbidity, prompting diagnostic investigations by the FHI, and following laboratory analyses to determine their cause.

3.2. Potential Origins of the Outbreak

Three possible origins of the ISAV affecting farmed Atlantic salmon in south west Shetland were considered. (1) It may have evolved spontaneously from an avirulent wild type virus such as HPR0 (Nylund et al. 2003), (2) it could have been introduced with imports from abroad, or (3) the 1998/9 virus might have persisted undetected.

Evolution of virulence in ISAV is considered to involve a simple deletion mutation in the hypervariable region (Krossoy et al. 2001; Nylund et al. 2003). Although individual sites were fallowed following the 1998/9 outbreak, MA 3a was not synchronously fallowed and therefore, at the MA level, was considered to be continuously stocked. This could allow virus to circulate over many generations, facilitating the emergence of virulent strains (Murray and Peeler 2005). Other pathogens including salmonid alphavirus (SAV), infectious pancreatic necrosis virus (IPNV), *Renibacterium salmoninarum* (the agent of bacterial kidney disease, BKD), and sea lice, had built up in MA 3a. These intercurrent infections would stress the fish potentially making them more susceptible to ISA infection. The MA is treated as 6 regions in the Code of Good Practice for Finfish Aquaculture (CoGPWG 2006) and

use of such small areas for management may be inappropriate for prevention of ISA emergence (Beattie et al. 2005).

ISAV could have been introduced with imported batches of ova or movement of equipment. Clinical ISA has been controlled in the Faeroes at great cost and in the initial absence of good biosecure practices, but continues to be present in Canada and Norway. Scottish contacts with Norway are much greater than with Canada. There is no evidence of foreign imports of live fish or dead fish for processing in the SPP and therefore there is no evidence MA 3a could have been exposed to ISAV via this route. Vessels servicing farms are locally based (see later section) so would not have contact with Norwegian fish. Norwegian ova were used to produce some of the smolts that were stocked to the index site A. However, the epidemiology of ISA (not affecting sites that received fish from the index site) indicates virulent ISA likely emerged late in the fish production cycle and if so, virulent ISAV was not considered to be introduced with the ova. In 2007, 33 million Norwegian ova were supplied to 16 Scottish mainland and Western Isles hatchery sites, and these were subsequently distributed to numerous sea sites within Scottish waters, with no evidence of ISA apart from the Shetland outbreak.

It is possible that HPR0 was present in ova or fish products imported from Norway, or elsewhere, and careful consideration should be given to its evolution (Kibenge et al. 2009). Since HPR0 is already present in Scotland, with no association with clinical disease, (McBeath et al. 2009), in this case, such imports are currently considered as representing a relatively low risk of emergence. The risk would increase if HPR0 was common in imports relative to its indigenous prevalence and resultant increased prevalence of HPR0 increased the chance of an emergence. This is quite different from a country in which HPR0 and virulent ISAV were both absent (Kibenge et al. 2009).

It is improbable that the virus that caused the 1998/9 outbreaks could have persisted unnoticed for 10 years. The 1998/9 ISAV (1998-390) caused high levels of mortality so would rapidly have emerged as a problem on affected farms. It is unlikely such a problem would not have been noticed, although restricted outbreaks could have been misdiagnosed. Genetic evidence shows the virus at 2008-0982 was a European genogroup 1 isolate, while the 1998-390 virus belonged to genogroup 3 (Nylund et al. 2007) and mortality took longer to occur in fish experimentally challenged with the 2008-0982 virus relative to those challenged with the 1998-390 virus (K. Urquhart in prep.).

The source of the virus behind the outbreak remains unknown however it is valuable to consider potential sources of introduction, as an aid to the control of future outbreaks.

3.2.1. Genetic Epidemiology

A comparison of the haemagglutinin-esterase protein gene sequences of 2008-0982 and other EU-G1 isolates has been undertaken (Figure 4).

The analysis appears to cluster 2008-0982 with three EU-G1 isolates from Norway (AY973179, FM203284 and FM203285). However, this is poorly supported with a bootstrap value of only 53% and the analysis does not therefore identify the 2008-0982 isolate as being an inclusive member of any particular terminal subgroup. Previously obtained isolates similar to 2008-0982 have been identified from several geographical areas along the Norwegian coastline rather than a single locality. Closely related non-virulent strains of the virus (HPR0) also occur and the possibility that one of these has recently evolved a capability to cause disease cannot be discounted. We conclude that, while the sequence data are not inconsistent with a Norwegian origin of 2008-0982, they do not provide definitive evidence of this.

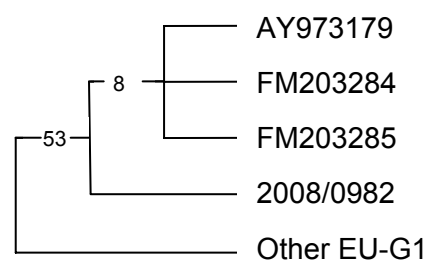


Figure 4 A maximum likelihood tree of 2008-0982 and other EU-G1 isolates.

The genetic evidence thus rules out that the ISAV was the same as the 1998-390 strain that caused the 1998/9 epidemic, but does not distinguish between a Norwegian or local Scottish origin.

3.3. Spread of the Outbreak

3.3.1. Movement of Live Fish

Movement of live fish from infected sites is a high risk activity for spreading disease, in the absence of good biosecure practices. The movements of fish within, into and out of, MA 3a have been used to develop a contact structure network (Munro and Gregory 2009) (Figure 5). This figure illustrates that there is no evidence to link ISA positive sites with the movement of live fish. The history of individual fish movements has not therefore been tracked in detail. However, there were substantial numbers of movements across MA 3a's boundaries.

Two movements of live fish occurred from the putative index site A and the two sites that received them were immediately put under official suspicion of being infected. Fish from one of these sites had already been harvested and processed therefore samples were not obtained (and the site is therefore not listed in Table 2). However, fish from the other (site g) were tested and found to be negative for ISAV. Since the movement of fish is high risk, virulent ISAV is likely to have been absent or, if present, at low levels at the time of the movement on 27 June 2008.

It is possible that if stocks on putative index site A were kept separate then only uninfected stocks might have been moved off. This would still argue for very low prevalence in the infected stocks at the time of movement, itself suggesting infection could at the earliest have occurred only shortly before the June 2008 movement.

There were no movements of live fish from any sites in MA 3a after 27 June 2008, with the exception of transport to SPP and LPP (detailed in section 3.3.2.c, including 'bus stop' harvests). There were, however, 12 seawater to seawater movements within MA 3a before 27 June 2008. There were also movements to and from sites in other MAs. Live fish were moved into MAs 4a and 2b and from MAs 4a, 3b, and 2c (Figures 5 & 6). If ISAV was not present before June 2008 these fish movements could not have spread the virus, however the sites out with MA 3a were visited by FHI to confirm the absence of ISAV, suggesting it was not present in this MA before June 2008.

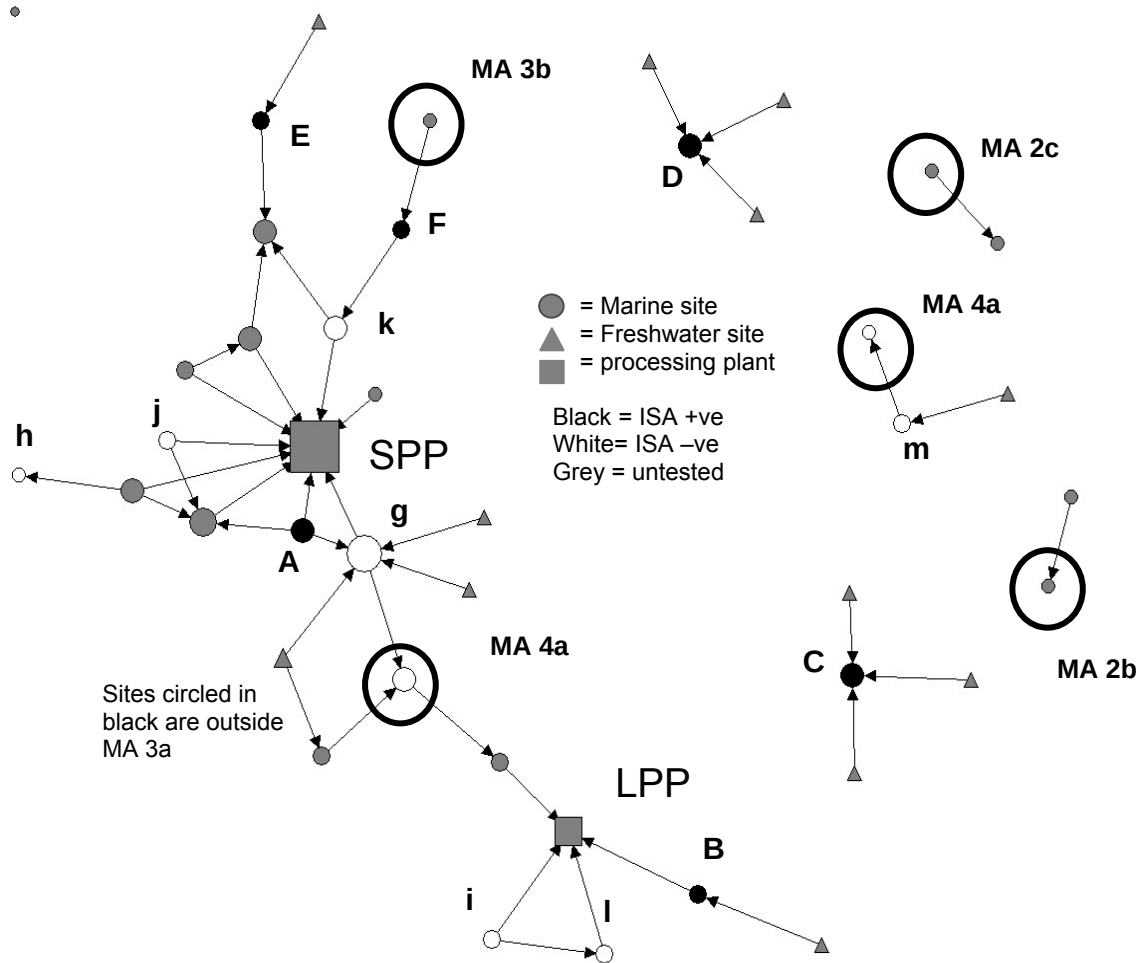


Figure 5 Structure of the network for movements involving salmon and trout in MA 3a. Circles are proportional to numbers of other sites contacted. Letters are site listed in Table 2; unlabelled nodes are sites not listed in the table (either harvested before site A was confirmed or in other MAs).

One movement occurred on 6 October from MA 3b to a site in MA 3a, and by this time ISA might have been present. This movement was from outside the affected MA, and therefore a far lower risk than a movement going the other way. A risk may still have existed of spreading infection with empty equipment moving to collect these fish (Peeler and Thrush 2009) but there was no evidence of transmission of infection to the source site.

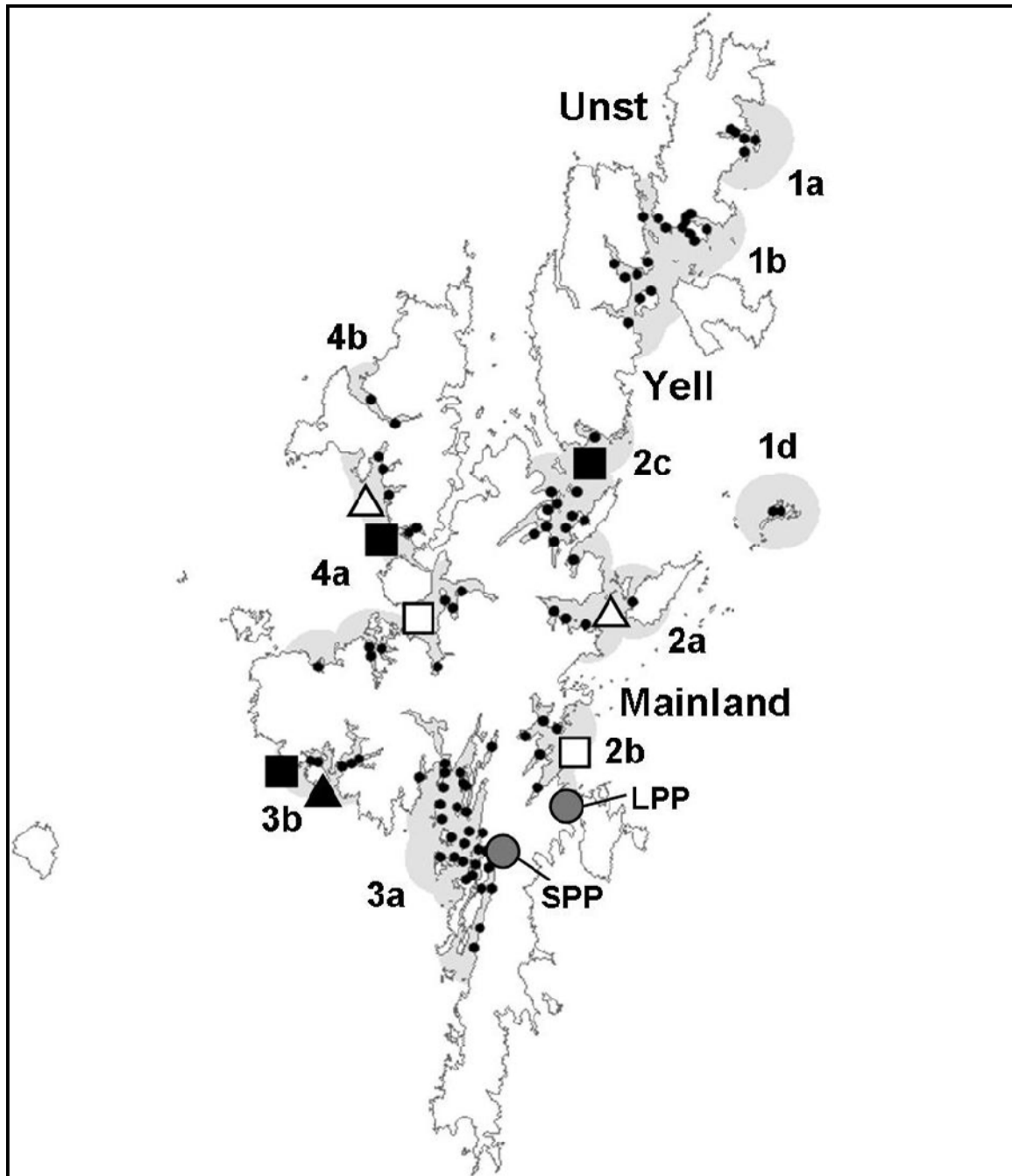


Figure 6 Locations of MAs in risk contact with MA 3a through movement of live fish or ‘bus-stop’ harvests to processing plants. ■ = live fish moved to 3a; □ = live fish moved from 3a; ▲ = bus stop to and from 3a; △ = bus stop to 3a; ● = fish farm and ● = processing plant. The shaded grey areas represent an amalgamation of tidal excursions around the fish farms.

All seawater to seawater movements are high risk in relation to the risk of disease spread and subject to assessment under the industry code of practice (CoGPWG 2006). Had the outbreak begun earlier than it appears it did and assessments did not consider the presence of ISA in the area (if it had not yet been confirmed),

infection could have been more widely spread (Figure 6). However, movements ceased in the last 6 months of 2008, before ISA would appear to have originated and there is no association between ISA outbreaks and the movement network (Figure 5). None of the 15 sites outside MA 3a that were tested returned ISA diagnostic positive results.

3.3.2. Harvest Operations

3.3.2.a. Processing Plants

Salmon from sites in MA 3a were primarily processed at either SPP within the area, and LPP in Lerwick on the east coast of Shetland. Both plants have advanced disinfection regimes and although it has not been independently confirmed that they were fully functional at all times Marine Scotland has found no evidence to the contrary, following risk assessments of the movement of live and dead fish to harvest (Fraser and Murray 2009). If disinfection was fully functioning then the associated risk of ISAV spreading was negligible according to the analysis of Jarpe and Karlsen (1997), although the risk would have been substantially enhanced if effluent disinfection was not employed.

The LPP did not receive fish from MA 3a between May and 11 December 2008, so it could only have been potentially exposed to infected material from December onwards. With notification of the presence of ISA on 2 January 2009 in MA 3a staff at LPP would have been aware of the risk of infection and biosecurity would have been an even higher priority.

This plant has facilities to hold live fish in adjacent marine cages. This is considered a high risk (Munro et al. 2003) as, due to their immediate proximity, such fish could potentially pick up infection released from the plant. Fish might then act as a continuous source of infection to visiting vessels, level of risk could increase with time on site. A similar situation is believed to have played a major role in the spread of ISA in 1998/9 (Murray et al. 2002).

The only site within 5 km of LPP was tested at the 150 fish level and placed under bi-monthly surveillance due to the potential risk should disinfection have failed (Jarpe and Karlsen 1997). However, this site showed no evidence of infection. All sites near the SPP which might have been at risk had disinfection failed, were already under enhanced surveillance as they were within MA 3a.

3.3.2.b. Transport Vessels

The transfer of fish to processing plants represents a series of risks for transmission of infection, particularly to other MAs (Munro et al. 2003; Fraser and Murray 2009). These risks can be minimised by processing within the MA (at SPP), however this is

not always practicable and fish have been, and are being, transported to LPP for processing. Two alternative practices were risk assessed. (1) Dead fish could be transported in boats to the Scalloway pier and then moved overland to Lerwick where they are unloaded into the LPP processing plant. Alternatively (2) fish could be shipped alive to LPP and pumped directly into the plant for processing.

The relative risks of these two processes have been reviewed using methods developed by Munro et al. (2003) and Fraser and Murray (2009). The greatest apparent risk from shipping live fish is transport out of the MA, however so long as the wells containing the fish on the vessels remain sealed and the vessels undergo disinfection (CoGPWG 2006), these fish represent little risk to the environment. Risks associated with the alternative on-site slaughter and movement of dead fish to LPP present several opportunities for infection to spread (at slaughter, at the Scalloway pier when unloaded, and at LPP when transferred). It is therefore probable that the risks associated with movement using well-boats is not greater, and may be lesser, than those associated with on site slaughter and overland transport.

Each of the processing plants had one dedicated vessel which serviced its own farms, except for a brief period in August 2008 (8-27) when a replacement vessel was used for LPP. This was before LPP had processed material from the control zone and so was of negligible risk. This made it relatively straightforward for Marine Scotland scientists to obtain copies of the vessel logs to undertake an analysis of movement patterns.

The SPP received fish from only one source outside Shetland, this being in Orkney. The fish were received from March to June 2008, likely before infection became established at index site A, and so these movements are very unlikely to have represented a risk, to be certain the Orkney site was inspected by the FHI. Harvests were taken from sites around Shetland, including the islands of Unst and Yell (Figure 6), to SPP. Most fish were entirely processed here, but some did go to packing stations prior to processing at SPP.

The spatial pattern of outbreaks, concentrated in a relatively small area close to the putative index site, is not consistent with well boats as a major vector of transmission. If it were, this would have resulted in cases throughout Shetland, reflecting the movements of these vessels. This different pattern of scattered cases of ISA infection, caused by well boat movements did occur in 1998 (Murray et al, 2002).

3.3.2. 'Bus Stop' Well Boat Movements

The vessel servicing SPP engaged in repeated use of 'bus stop' harvests, picking up a partial load of fish from one site then going to another to complete a full load en route to a processing plant. If the first site is infected, then infection might be transmitted to the second by this movement. These 'bus stop' harvests occurred on

at least 71 occasions in 2008 with some of these involving movements between MA 3a and other MAs (Figure 6). Seven 'bus stop' movements involved the putative index site A, these occurred during the September to November 2008 period when ISA may have been present (Figure 7). Movements in which the index case was the second site to be visited were of low risk, but those where it was visited first represented higher potential risk. One such visit involved a site in another MA, (MA 3b) (Figure 7). The level of risk depended on levels of biosecurity practiced by the operators of the well boats and time that fish remained on site after the visits. Due to these 'bus stop' activities being involved in harvesting operations, the sites may be quickly cleared and this is quite different from 'bus stop' deliveries involving smolts.

There were a further 5 'bus stop' harvests from MA 3a to 3b and 7 from MA 3b to 3a, excluding the harvests involving the putative index site (Figure 6). There were 2 harvests from MA 4a (North West Shetland) to 3a and also 2 from MA 2a (north east Shetland) to 3a. All these 'bus stops' involved sites around the mainland island of Shetland, but MA 2a is on the east coast, so there is a potential for 'bus stop' activities to result in long distance transmission. In this instance the movement was into, not out of, MA 3a so the risk of spread was lower.

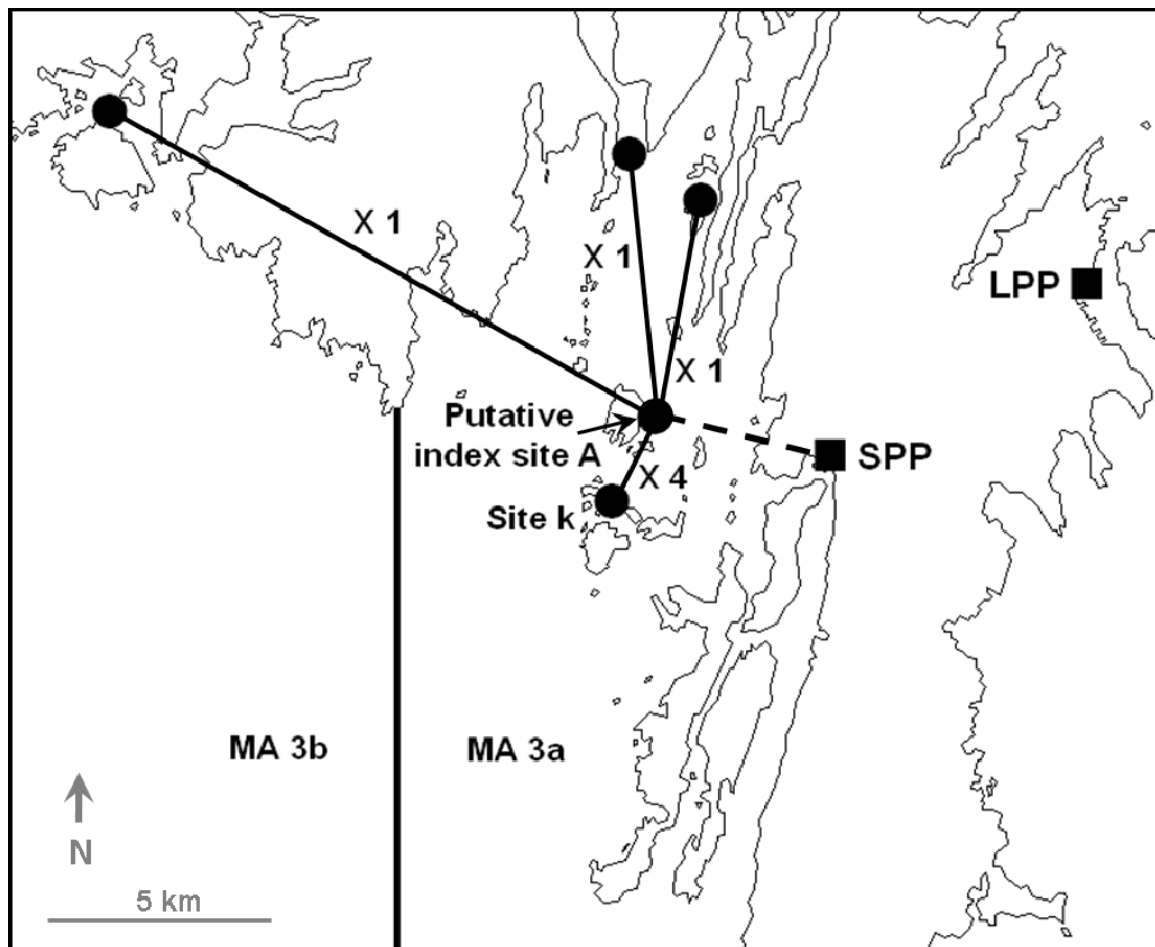


Figure 7 'Bus stop' harvests involving the index site A. ● = fish farms; ■ = processing plants; solid lines between fish farms represent 'bus stop' harvests; numbers refer to the number of 'bus stops' and the dashed line represents movements to the processing plant. In the 4 harvests involving site k, salmon were taken from here before the well boat visited the index site A. However, when fish were taken from the other 3 sites, including a site in MA 3b, the well boat had visited index site A first.

The other vessel's log is less clear so we do not know if such activities were the normal practice. Only sites visited are logged (sometimes only the company name without a specific site location was recorded) and not whether the vessel returned to the processing plant between the site visits. However, this vessel was not active in the area for most of the period before ISAV was detected.

There is no evidence that infection was spread by the movement of these vessels out with the controlled MA, although this was clearly the case in 1998/9 (Murray et al. 2002). Improved disinfection at processing plants and for vessels may have been important in this. However, the practice of 'bus-stop' deliveries remains a risk and is contrary to the CoGP which stipulates stage 2 disinfection when well boats leave an existing site to start work at another site (CoGPWG 2006).

3.3.3. Vertical Transmission

3.3.3.a. Movement of Smolts

Some of the fish on the index site A were reared from Norwegian ova. These could be the source of infection, however given that ISA seems to have appeared on site late in the production cycle it is unlikely that infection was within the ova via vertical transmission. In 2007, 33 million Norwegian ova were supplied to Scotland with no evidence of ISA, apart from the Shetland outbreak.

Smolts were imported into MA 3a from various producers around the UK (Shetland, the Western Isles, mainland Scotland and England (Figure 8). Essentially ISA is a marine salmonid disease with almost all reported cases being from marine salmon, and those from freshwater involve some contact with sea water (Jarp and Karlsen 1997). Due to this marine nature, and there was no reason to suspect ISA in the UK, these freshwater sources are very unlikely to represent risks of ISAV infection to Shetland.

The pattern of contacts in Figure 8 represent a network by which ISAV could theoretically have spread throughout the UK, as pathogens might be transported with equipment going to the smolt producers (Peeler and Thrush 2009). However, the risk appears negligible because the overall risk consists of four very low risk steps that combine to produce this negligible risk: (1) Only empty transporters moved from MA 3a to the remote sites, so risk of transfer to these sites is extremely low; (2). These sites are freshwater, while ISA is essentially a marine disease, except occasionally in broodstock recently moved to freshwater (Lyngstad et al. 2008). Therefore infection is unlikely to spread even if ISAV reached the smolt producers; (3) Sometimes there was no direct contact between the sites and well-boats, with smolts being moved to the well-boats overland, so contact between MA 3a and the smolt producers was often broken; (4) The freshwater to seawater movements into MA 3a all occurred before June 2008 and therefore before infection was established at the putative index site A.

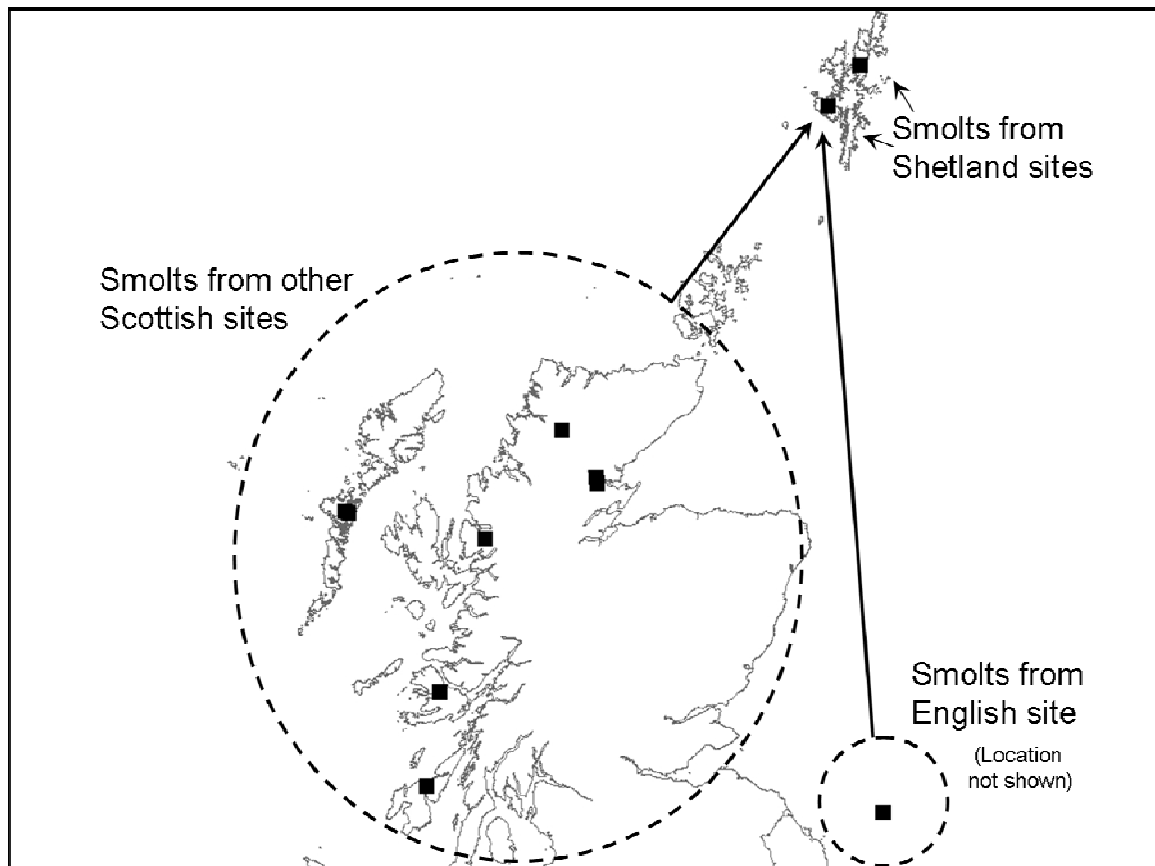


Figure 8 Sources of smolts moved into MA 3a within the year 2008. ■ = source fish farms.

Previously ISAV has been found in wild freshwater salmonids using PCR (Raynard et al. 2001) however the spread appears limited. The widespread sourcing of smolts is probably of more concern for the spread of diseases such as BKD or infectious pancreatic necrosis (IPN) that exist in both marine and freshwater stock (Murray 2006). Such diseases may have the potential to spread around the UK, and as such they should be monitored.

3.3.3.b. Vertical Transmission and Movements of Ova from Shetland

There is inconclusive evidence for vertical transmission of ISAV (Lyngstad et al. 2008; Vike et al. 2009). If it could occur via the movements of ova, there is the potential risk of the spread of ISAV within Scotland and beyond via Atlantic salmon broodstock reared in Shetland. The only broodstock site on Shetland is in MA 1b on the island of Unst (Figure 6).

There were no movements of live fish out of the control and surveillance zones to sites outside mainland Shetland, and none at all out of MA 3a after June 2008. As such it is reasonable to conclude that there was negligible risk of transmission to sites on Unst as a result of live fish movement.

Harvest vessels from SPP visited sites belonging to the company that owned the broodstock site in July, August and September 2008, times when the plant could potentially have been processing infected material from MA 3a. It is therefore possible that there may have been contacts with sites within the MA from which the broodstock originated. This risk is probably negligible if disinfection at the SPP was correctly implemented.

The evidence for vertical transmission of ISAV is ambiguous (Lyngstad et al. 2008; Vike et al. 2009) and the broodstock fish exhibited no signs of excessive mortality when visited by the FHI. The eggs were disinfected on 3 occasions: immediately following stripping when “soft”; before transferring and at the receiving site in mainland Scotland. In these circumstances, and given the extremely low risk that the broodstock were exposed in the first place, the overall risk of vertical transmission must be regarded as negligible (Peeler et al. 2007) in the presence of good biosecurity practiced on all occasions.

If evidence showed that the risks posed by these broodstock were considered to be non-negligible, the same risk would apply to all broodstock sites sharing a MA with a site that had used a processing plant that had processed fish from any MA in which ISA was present. This is a stricter definition of risk than is imposed on imports from Norway, a country where ISA is endemic. It would be contrary to current evidence to impose such a restriction on Scottish smolts without equally imposing it on imports. The investigation has shown no association with Shetland broodstock/ova and the Shetland Islands ISA outbreak.

3.4. Wild fish

As part of the epidemiological survey wild fish were screened for ISAV using qPCR (Wallace et al. 2009). Two discreet field sampling trips were undertaken by Marine Scotland staff, the first for marine and, the other for fish caught in freshwater with the

specific aim of sampling salmonids. Saithe from within an infected salmon farm were also sampled opportunistically.

A total of 1196 wild marine fish from 12 species were caught from the 3 trawl areas within MA 3a (Figure 2 and Table 4). Area (1) was in the vicinity of the then actively infected site B making this a good time to sample wild marine fish and 446 from this area were sampled. The kidney and gill samples were thought most likely to carry ISAV so these were initially screened. All these samples tested negative for ISAV by qPCR and for this reason the brain and heart tissue were not screened.

Table 4

Wild marine fish sampled for ISAV in February 2009. Numbers refer to the trawl locations (Figure 2).

Species	Papa – Hildasay (1)	North Havra – Fora Ness (2)	Scalloway Deeps (3)
Long rough dab (<i>Hippoglossoides platessoides</i>)	150	50	
Saithe (<i>Pollachius virens</i>)	30	10	30
Whiting (<i>Merlangius merlangus</i>)	35	150	110
Common dab (<i>Limanda limanda</i>)	100	150	
Norway pout (<i>Trisopterus esmarki</i>)		150	
Herring (<i>Clupea harengus</i>)		30	
Plaice (<i>Pleuronectes platessa</i>)	55		
Short-horn sculpin (<i>Myoxocephalus scorpius</i>)	70		
Atlantic cod (<i>Gadus morhua</i>)	4		25
Flounder (<i>Platichthys flesus</i>)	2		
Haddock (<i>Melanogrammus aeglefinus</i>)			30
Lesser Argentine (<i>Argentina sphyraena</i>)			15
Total	446	540	210

Results from the freshwater sampling are presented in Table 5; refer also to Figure 2. In total, 216 fish caught from the freshwater environments were sampled comprising 178 salmonids and 38 non-salmonids. The later sampling date was chosen to allow the freshwater temperatures to warm up. From previous personal experience these higher temperatures make the fish more active thus increasing the chances of catching them. Additionally there would be a greater chance of catching migratory sea trout returning to freshwater at this time of year.

Table 5

Sampling of fish caught in freshwater for ISAV in March 2009. Numbers refer to site locations (Figure 2).

Species	Stromfirth burn 23 rd March (4)	Stromfirth burn 24 th March (4)	Weisdale burn (5)	Scalloway burn (6)
Atlantic salmon parr (<i>Salmo salar</i>)	30	34		
Brown trout (<i>Salmo trutta</i>)	2	1		
Sea trout (<i>Salmo trutta</i>)	5 (3 escapes)	2		
Brown/sea trout			22	82
European eel (<i>Anguilla anguilla</i>)	22	3	8	1
3-spined- stickleback (<i>Gasterosteus aculeatus</i>)	2			1
Flounder (<i>Platichthys flesus</i>)	1			
Total	62	40	30	84

This sampling also coincided with an active infection in site C, which was in the process of being harvested. Kidney and gill samples from all these fish were screened using qPCR and found to be negative. Wild salmonids are particularly likely to carry ISAV infection (Nylund and Jakobsen 1995; Raynard et al. 2001) so these negative results are of particular importance.

Two saithe taken from within an infected cage at the third ISA confirmed site (C) were individually sampled. Gill and kidney samples were screened for ISAV using qPCR with both gill samples testing positive (2/2). This small sample size indicates a low population of saithe in the infected cage however those that were present had been exposed to infection. Saithe are resistant to ISAV infection and quickly clear the virus (Snow et al. 2002; McClure et al. 2004a) so they are unlikely to be effective vectors. The qPCR results probably reflect exposure of the gills to virus laden water rather than active infection.

With the exception of the saithe taken from within a clinically infected farm wild fish, even salmonids, showed no sign of infection with ISAV. We conclude that wild fish were of low, if any, importance in the initial spread of the virus. This does not rule out the possibility that prolonged exposure would result in their becoming infected, however there has been, to date, no evidence of mortality or clinical ISA in wild fish populations from any ISAV infected country.

3.4.1 Seal attack and Associated Activity

Mortality records collected from ISA affected farms later in the outbreak attributed very high levels of mortality to seal attacks. This self reporting is not independently confirmed, but high levels of attacks on sick fish could allow for escapes to occur and potentially the spread of infection. Such fish can travel large distances and collect near farms where they can obtain waste feed (Dempster et al. 2009; Uglem et al. 2009). Grey seals (*Halichoerus grypus*) are known to travel long distances at 75-100 km d⁻¹ (McConnell et al. 1999), but the virus is destroyed within the digestive system, and other associated risks are deemed low.

3.5. Hydrodynamics and Spread

Proximity to an infected site could be a risk factor for a variety of reasons, but transmission through the water is strongly indicated. Movement through the water is the only explanation for the downstream affect found by Gustafson et al. (2007), and shared contacts are explicitly included by shared ownership in the model of Scheel et al. (2007). Hydrodynamic spread is believed to be important in the epidemic currently affecting Chile (Mardones et al. 2009).

As a result of this hydrodynamic transport one of the principle controls on ISA in Scotland is based on tidal excursion modelling and the existing MA 3a was defined by this model. The model was developed during the ISA outbreak in 1998/9 and uses simple oceanographic assumptions to define a distance over which ISAV could be moved by tidal currents (Stagg 2003). The distance is 3.6 km in Shetland (the distance is twice this on mainland Scotland where tidal ranges are greater). Sites falling within this distance are included within the control zone, while a surveillance zone is set up for all sites around the control zone until a separation distance is reached where tidal excursion zones do not overlap. This is an effective way of deriving zones on which controls can be imposed.

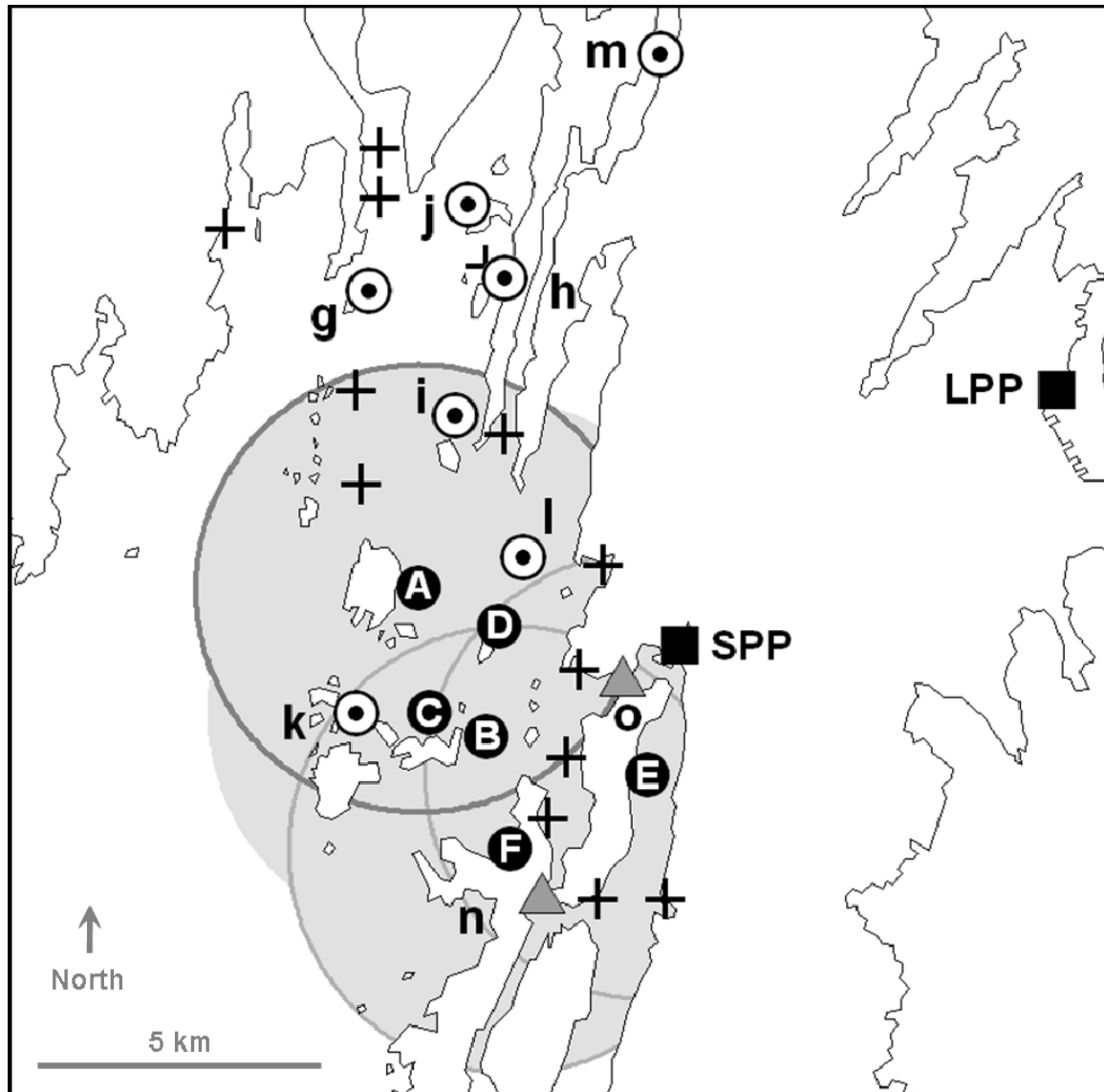


Figure 9 Map of sites involved in the outbreak. The letters refer to the farms listed in Table 1: + = farms depopulated prior to the outbreak; ● = ISAV positive; ⊙ = ISAV negative; ▲ = non-susceptible species; ■ = processing plant. Sites B, C and D are within 3.6 km of site A; E and F are slightly outside this but well within overlapping tidal excursion distances.

Simple modelling indicated ISAV was unlikely to be transported beyond the tidal excursion zone due to its rapid decay, meaning longer-term hydrodynamic movements were unlikely to be relevant (Murray et al. 2005). All affected sites, apart from the putative index case, were within overlapping tidal excursion distance of an infected site and most were within a single radius of an infected site (Figure 9). The sites E and F are slightly more than 1 tidal excursion distance, but within overlapping excursion distances: this overlapping distance is the standard model used to identify sites at risk in both this and the 1998/9 outbreak. Both sites are within the excursion radii of sites within 1 radius of A, and so infection may well have spread indirectly.

Site F is connected by exposed open sea to site A and therefore could have been exposed to virus transported via a short-term current. Infection was very slow to develop at site F, which might indicate exposure to a very low dose of virus from A. Site E is within a tidal excursion of SPP, so had there been a failure of disinfection it is possible that this could, also via hydrodynamic transport, be the source of infection. Although prevailing offshore currents may be stronger than coastal currents, the significant separation distance between MA 3a and its neighbours means water-borne transmission to other MAs is unlikely.

Local intra-company contacts might explain the local spread and shared ownership is a risk factor for ISA in Norway (Scheel et al. 2007). In Shetland ISAV has been detected in sites belonging to 4 separate companies (Table 2) and there was no movement of fish between affected sites (Figure 5). Sites B, C and D belonged to one company, so intra-company contacts to B and/or C is a possible explanation of spread. The FHI found these sites (B, C and D) had site-specific equipment which was not moved off site and site dedicated boats. Furthermore any such intra-company transmission would apply to cases that occurred months after the putative index case had been confirmed and so biosecurity should have been a high priority. No transmission occurred to the other sites belonging to the company that owned site A, even although ISAV had not been detected, and heightened biosecurity was not in place.

The highly clustered nature of the outbreak, all cases within a few km of the putative index case argues for environmental transmission. The pattern does not fit with transport of fish, harvest vessel movements or company ownership. It is possible that wild fish could have spread infection, but in the absence of positive results (Wallace et al. 2009) and the established association of ISAV transmission with water movements, hydrodynamic transmission of infection is the most likely driver of this outbreak's spread.

3.6. Limitation of the Outbreak

The 2008/9 ISA outbreak appears to have been localised to a relatively small area, although it spread widely within that area. This is unique, with other outbreaks including the 1998/9 Scottish outbreak becoming widespread rapidly (Murray et al. 2002). Similarly outbreaks in Chile spread from region X to regions XI and XII and multiple clusters of local spread developed (Mardones et al. 2009). In Norway a variety of different ISAV genogroups were found scattered along the coast, arguing that long-distance spread events are fairly frequent (Lyngstad et al. 2008). The ISAV has also spread between the different salmon farming islands in the Faeroes archipelago. While infection demonstrates a pattern of transmitting most easily in clusters, with relative risk about 10 times greater (Mardones et al. 2009), the virus frequently is transmitted over long distances to form new clusters or isolated outbreaks.

The spread of the 2008/9 ISA outbreak appears to have been dominated by water movement. This is in contradiction to the 1998/9 outbreak, for which vessels and movement of fish between different areas of the country spread ISA to virtually all areas of Scotland in which salmon were farmed (Murray et al. 2002). A variety of routes of spread (seawater-to-seawater movement of live fish, harvest operations and wild fish movement) were identified, but none were associated with the spread of ISAV. It appears that strategies developed as a result of the 1998/9 outbreak (Anon 2000; CoGPWG 2006) had been effectively applied. This also corresponds to results from Norway where Aldrin et al. (2010) found shared ownership was a small risk factor compared to close neighbours. However, practices were identified which had the potential to increase the spread of infection; this underlines the need for industry to be vigilant in the application of good biosecurity practices.

The seawater-to-seawater movement of fish did not occur in late 2008, but such movements had occurred earlier, so had the outbreak occurred earlier it could have been more widely spread. Harvest vessels that played an important role in the 1998/9 spread may have avoided contamination, because effective disinfection appears to have been in place at the processing plants that received fish from sites in the MA. However, inappropriate practices of bus-stop harvesting were occurring frequently and so the risk of transmission by well boats did exist, even between MAs, involving risks in the absence of good biosecurity (Fraser and Murray 2009).

Wild and escaped fish caught early in the outbreak were found to be negative for ISAV (Wallace et al. 2009). As wild fish are established vectors of ISAV (Nylund and Jakobsen 1995; Raynard et al. 2001), the patterns of movement of fish between sites (Uglen et al. 2009) and the exposure of wild fish to viruses in the vicinity of infected farms, as illustrated by IPNV (Wallace et al. 2008) there is the potential for wild fish to transmit infection, where it remains on farm sites.

Smolts were supplied into the MA area at the Great Britain level; this was not associated with spread of ISAV and probably represents a greater risk of spreading diseases with a freshwater component, such as IPN (Murray 2006). However in 1998/9 marine site-to-site movements occurred over 100s of km and contributed to spread of ISAV at the Scotland scale (Murray et al. 2002), these movements continue, but at the regional scale (Figure 6). Ova are imported from several countries, including Norway, and so although we have no evidence of this having occurred in the UK the potential for vertical transmission should continue to be investigated (vertical transmission in Chile is currently suspected (Vike et al. 2009)).

Blue mussels (*Mytilus edulis*) are abundant in Scottish waters and these adsorb and inactivated ISAV rapidly, so are not likely to act as reservoirs (Skar and Mortsen 2007). Sea lice may potentially act as vectors of infection (OIE 2009). Lice from a treated or culled site might drop off their hosts and be transported to neighbouring farms in large numbers so may enhance spread at the local level.

Other routes of spread may also exist at the regional level, particularly in the absence of good biosecurity, so long as there is a source of virus. These may include other vessels such as ferries or fishing boats, harvest and grading equipment, divers or other staff visiting sites, birds or other predators. Even hydrodynamic spread between MAs is possible, since current velocities can be large. Since a MA cannot be sealed off, the possibility of spread to other MAs can be reduced but cannot be eliminated so long as infection (and especially disease) is present.

3.6.1. Removal Policy

Rapid removal of salmon from confirmed infected sites and the implementation of good biosecurity practices were factors believed to have played a critical role in control of the 1998/9 outbreak (Stagg 2003; Mardones et al. 2009). Aldrin et al. (2010) found a history of infection was not a risk factor for ISA in Norway, so depopulation is effective at removing the virus. Other countries have allowed delayed removal or require mandatory removal of fish only after mortality reaches a trigger level. In Scotland site-level depopulation is carried out, even if ISA is restricted to a few cages, but in other countries depopulation may be applied only to cages with clinical disease. McClure et al. (2004b) showed that the prevalence of ISAV in non-moribund fish was the same irrespective of whether or not mortality occurred in a cage provided there was mortality on the farm; this observation supports site-level culling.

In the Shetland 2008/9 outbreak depopulation was carried out within 7 weeks of confirmation. The putative index case site A had already been harvested for 13 days at the time of confirmation. The other cases were harvested 35 (site B), 19 (site C), 45 (site D), 6 (site E) and 26 (site F) days respectively after confirmation (mean 26 days = 3.7 weeks). This may be compared with a mean of 21 weeks in Chile (Mardones et al. 2009), where ISA became widespread within the industry. However, 3.7 weeks is marginally longer than the 3 weeks removal time achieved in 1998/9 (Stagg 2003). Identification of a practical rapid and biosecure method of removal delayed the clearance of fish from site B. However, once a suitable method was identified, the site was depopulated in a 9 day period. The sites C and E were rapidly depopulated; site E being a small experimental facility. Depopulation of site D was slower, by this time only 2 other sites remained populated with susceptible species (salmon) within the MA 3a, and one of these was already infected so potential for local spread was limited. However, MAs can never be 100% isolated and so depopulation was eventually achieved 6.4 weeks after confirmation.

The increased shedding from clinically affected fish on confirmed sites increases the risk of horizontal transmission and the possibility for spread, even between MAs. This would increase the longer fish are left on site. The risk is more than linearly increased with time because farm-level prevalence of infection increases and clinical disease is increasingly likely. The potential for escapes in infected populations,

especially after seal attacks, increases the risk of fish movement between MAs. The consequences of infection escaping the MA are very large and therefore the principles of risk analysis (non-negligible probability × high consequence) indicate that leaving infected fish on site is a serious risk (Peeler et al. 2007).

Many of the licensed salmon sites were not populated even before the outbreak, with only 12 stocked sites at the time of confirmation. Five uninfected sites in MA 3a were depopulated as a result of normal harvesting operations within 4 weeks of the outbreak being detected. This fortunate coincidence greatly reduced the potential susceptible population within the MA. Following depopulation of the confirmed sites, which are fallowed for 6 months and suspect sites which are fallowed for 3 months, the control area will be subject to a co-ordinated fallowing period of 6 weeks before the processes of assuring the restoration of ISAV freedom can begin. Confirmation of ISAV freedom will involve a 2 year period of surveillance and diagnostic testing of all the stocked sites in the surveillance zone, after which MA 3a can be adsorbed back into the ISAV free zone that incorporates the rest of Great Britain.

3.6.2. Subclinical Infection

The case of site F (and possibly D) suggests subclinical infection may persist undetected on sites for months. Alternatively there could be an environmental reservoir, if so longer fallowing may be required for eradication, or an independent evolution of virulence may have occurred (viral sequencing is planned, to answer this question); it is unlikely that there would be two random emergence events in MA 3a within a year, and none elsewhere in Scotland in a decade.

The MA wide movement restrictions and fallowing of sites, following clearance of confirmed infected sites, help manage the risk from undetected subclinical populations (provided there is at least one known ISA case in the area). Even if such unknown infection exists fish will not be moved off and shedding will be limited in the absence of clinical disease (Gregory et al. 2009). However, infection may still spread e.g. with escapes.

Synchronised fallowing of only control zones rather than whole MAs may represent a serious risk to attempts to stamp out infection. Subclinical infection could persist in other parts of the MA and flare up, and involve repopulated sites. There is a good case for area wide fallowing, post harvest, but this is not currently a requirement before restocking. In the case of this outbreak prolonged synchronous fallowing was planned and did occur throughout the winter.

Diagnostic sampling in the absence of clinical signs may be required to detect ISAV infection. However, if transmission risk is minimised by MA-wide controls then ignorance of the location of infection may not be too serious. It might be that

diagnostic screening of all populated sites in the MA may reduce the risk from not carrying out synchronous fallowing.

The most serious implication of prolonged periods of asymptomatic infection occurs when no known cases of infection exist, and hence the area is not subject to movement restrictions or to enhanced surveillance. It is for this reason that movements of fish between different MAs should be minimised in the absence of known cases of ISA. Following depopulation of the confirmed sites, which are fallowed for 6 months and suspect sites which are fallowed for 3 months, the control area is subject to a co-ordinated fallowing period of 6 weeks before the processes of assuring the restoration of ISA freedom can begin. In this case a longer fallowing was achieved because following the clearance of the last site on 25 November 2009 restocking did not commence until spring 2010, so a 3-4 month fallowing occurred.

3.6.3. Practices Contributing to the Potential Future Re-Emergence of ISA

Virulent ISAV might re-emerge from local avirulent HPR0 or be imported (Murray and Peeler 2005). The HPR0 strain was found on 3 of 30 anonymously surveyed Scottish salmon farms (McBeath et al. 2009); however its presence is not associated with clinical ISA disease. Furthermore ISAV has been detected from wild salmonid fish in Scotland (Raynard et al. 2001; Cunningham et al. 2002). It is therefore unlikely that the eradication of HPR0 from Scottish salmon farms can be cost effective although co-ordinated fallowing will help keep prevalence low. However, as long as HPR0 is present there remains the potential for ISA disease to re-emerge (Cunningham et al. 2002). Utilising local well boats for local farm activities such as harvesting is a good practice and should continue. However, it is possible that ISAV could be imported with other vessels or even escaped or wild fish. It is also possible there is a risk of ISA emergence due to use of imported ova from Norway, however opinion on vertical transmission is divided. Since eradication of HPR0 is not practicable and contact risk for import of foreign virus is low, there is little that can be done to further reduce exposure risk, unless the risk of vertical transmission is significant. Since there is no evidence that the 1998/9 or the 2008/9 ISA outbreaks were linked to vertical transmission, this means that in the future there is a high likelihood that ISA will recur even if the import of ova ceased (although the time taken for this to occur might be increased).

In the event that ISAV is introduced, the emergence of disease is encouraged if the virus can circulate in populations of fish at high levels for long time periods. This circulation is broken by fallowing. Fallowing is currently carried out at the farm level but for it to be more effective in breaking local infection cycles, MAs should be synchronously fallowed. The operators of the fish farms within MA 3a were progressing towards a synchronous fallowing strategy however this had not yet been implemented. Due to the fact that ISAV seems to have persisted undetected on site F for many months, in spite of frequent visits by FHI, anything other than

synchronous fallowing could allow such hidden infection to transmit to newly input smolts on neighbouring sites. The emergence of multiple disease problems at the same time (sea lice, SAV and BKD) in addition to ISA further illustrates the need for synchronisation. The 1998/9 outbreak began in a site containing multiple generations of fish (Stagg 2003) underlining the need for fallowing.

If ISA were to re-emerge, it is imperative that there is a quick response by industry and the regulatory authorities; this requires rapidly detecting the presence of ISAV. Neither the 1998/9 nor the 2008/9 index cases were detected by active surveillance. There simply can never be enough FHIs to visit sites frequently enough to detect ISAV within a short time from an outbreak beginning, in the absence of information to target particular sites. However, passive surveillance has also been disappointing with ISA only reported in 1998 once high levels of mortality had begun and the virus was widely dispersed (Murray et al. 2002) and in 2008 mortality was ascribed to high sea lice burdens. In 2008 inspectors did target the putative index site following up reports of mortality and took samples for diagnostic screening, even although there was an alternative explanation for the mortality. This use of intelligence-led sampling indicates that reporting of unusual mortality even if it is 'explained' could be a valuable tool to more effectively targeting Marine Scotland's resources. There is provision for an element of intelligence led surveillance in Scotland's current risk based surveillance programme.

Once ISAV was detected on the putative index site active surveillance of the remaining sites in MA 3a, and sites in other MAs identified as having potential contact with MA 3a, was effective in detecting further ISA cases. This is because the risk-based targeting of surveillance effort allowed these sites to be frequently visited. Site E was detected by passive surveillance, with suspicion of ISA being reported by a veterinarian.

A highly important factor in the limitation of ISA in 2008/9 was the MA structure developed following the 1998/9 outbreak (Stagg 2003). MAs must be epidemiologically appropriate, and the spread of ISAV between smaller MAs that were defined in the Code of Good Practice (CoGPWG 2006) showed these to be ineffective. A MA can never be completely biosecure and so infection may spread while infected fish remain, even after movements of live fish have been prevented. Movements of live fish between MAs (Figure 6) should not be undertaken, even in the apparent absence of infection. This is because infection can be spread if there is any delay between emergence and detection as occurred in 1998 (Stagg 2003).

Another important factor was the disinfection of processing plant effluent and the disinfection and sealing of well boats (CoGPWG 2006). All waste from processing plants was disposed of by approved methods. From investigation, there is little evidence to associate well boats with the spread of ISAV, unlike in 1998/9 (Murray et al. 2002) and this has been a major achievement in reducing Scotland's vulnerability

to ISA outbreaks. However preventing the risky practice of bus-stop deliveries could further reduce the risks.

Synchronous fallowing of MAs is likely to substantially reduce the risk of the emergence of ISA, however the risk cannot be eliminated. Epidemiologically appropriate MAs have been successful in confining the virus. It is likely impossible to prevent hydrodynamic spread within MAs, while open culture net-pens are used to farm salmon. Movement of fish between sites in different MAs and bus-stop deliveries are identified as risky practices that should be prevented or if this is unfeasible then carefully controlled. Disinfection of processing plant effluent and the use of local dedicated well boats are good practices that should be encouraged.

4. Conclusions

An outbreak of ISA has occurred in Scotland. The outbreak is of unknown origin, but was not related to the ISAV that was responsible for the 1998/9 outbreak. Whether ISA evolved from a local avirulent HPR0 virus or was imported via an unknown route, such a re-emergence may be repeated and so, even on eradication, complacency cannot be allowed in aquaculture biosecurity. Policies such as co-ordinated fallowing of areas and risk based surveillance may deny emergent or introduced virus a chance to establish.

A rapid response to the outbreak by Marine Scotland was possible, because of rapid detection of infection. Detection at site A occurred as a result of intelligence from an unconfirmed source in relation to alleged high sea lice mortality in Shetland and inspectors following up general health problems, not specifically targeting ISA. This observation justifies the need for Marine Scotland to investigate reports of mortality, even if ascribed to a non-notifiable cause, and indicates the limits of passive surveillance for notifiable diseases in the face of multiple potential causes of mortality. Intelligence led surveillance plays an important role in Marine Scotland's current risk based surveillance programme. Active surveillance is of limited effectiveness unless the limited available effort can be targeted in a risk-based manner. Even then, subclinical infection may go undetected and so there may be a case for regular diagnostic sampling (instead of just the current one off sample) in the absence of signs of ISA if sites are likely to have been exposed to infection.

Fallowing is important in eliminating ISAV and other diseases and in preventing their emergence in the first place. Currently there is no requirement to fallow whole MAs synchronously, only control zones and this practice may allow subclinical infection to persist. Surveillance zones within a protected area do fallow for 6 weeks asynchronously, which will have a positive effect on minimising re-emergence of disease. Fortunately, all sites in MA 3a were fallowed for the whole winter of 2009/10.

The outbreak was confined to a relatively small area, although the virus spread easily within that area. The strategic breaking of the industry into biosecurity-determined management areas by Marine Scotland Science for the purpose of disease control appears to have been effective at controlling ISAV. Isolation has been maintained because of an absence of seawater-to-seawater movements of fish in the critical period and the disinfection of processing plant waste and well boats. Depopulation of confirmed sites prevents these from acting as sources of infection.

The industry has played a key role in the design of these effective disease control measures (Anon 2000; CoGPWG 2006); this has been done in collaboration with the Scottish Government; at significant financial cost to industry and the government.

There have been serious failings in these controls, seawater-to-seawater movements were quite extensive in the first six months of 2008, and if an outbreak had occurred at that time, ISAV could have spread much more widely, at least throughout Shetland. Similarly, harvest vessels practised 'bus-stop' harvests, also potentially risking spread at the regional scale. Some depopulation has been delayed owing to technical or other reasons, allowing large numbers of diseased fish to remain in the environment for considerably longer than the mean 3-4 week period. These detected failings suggest industry practice could be improved to further reduce the risk from any future ISA outbreaks, especially before infection was detected, and also reduce the probability of outbreaks occurring in the first place. These failings aside, industry behaviour combined with the speedy response of Marine Scotland, has played a key role in the confinement of ISAV relative to spread in other countries, or indeed Scotland in 1998/9.

The disease control mechanisms that appear effective at controlling ISAV in Scotland help to control other diseases. However, the contact structure involving freshwater sites spreads throughout the UK, compared to the networks of marine site-to-site movement (local and limited) and of harvest (regional). As such, diseases with freshwater spread are likely to be more difficult to control. Similarly more robust pathogens might transmit over greater distances and so be more likely to transmit between MAs. With suitable modifications, the system of local MAs with associated good biosecurity measures that appears to have been successful in the control of ISA, and refined in the light of epidemiological information, may be applied to the control of a range of diseases.

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Appendix 1 Details of Diagnostic Methods: Eann Munro

As detailed in the Office international des épizooties (OIE), Manual of Diagnostic Tests for Aquatic Animals 2009, the diagnosis of ISA is based on clinical and pathological findings as well as detection of the agent. The diagnostic methods are summarised in this appendix 1's figure 1.

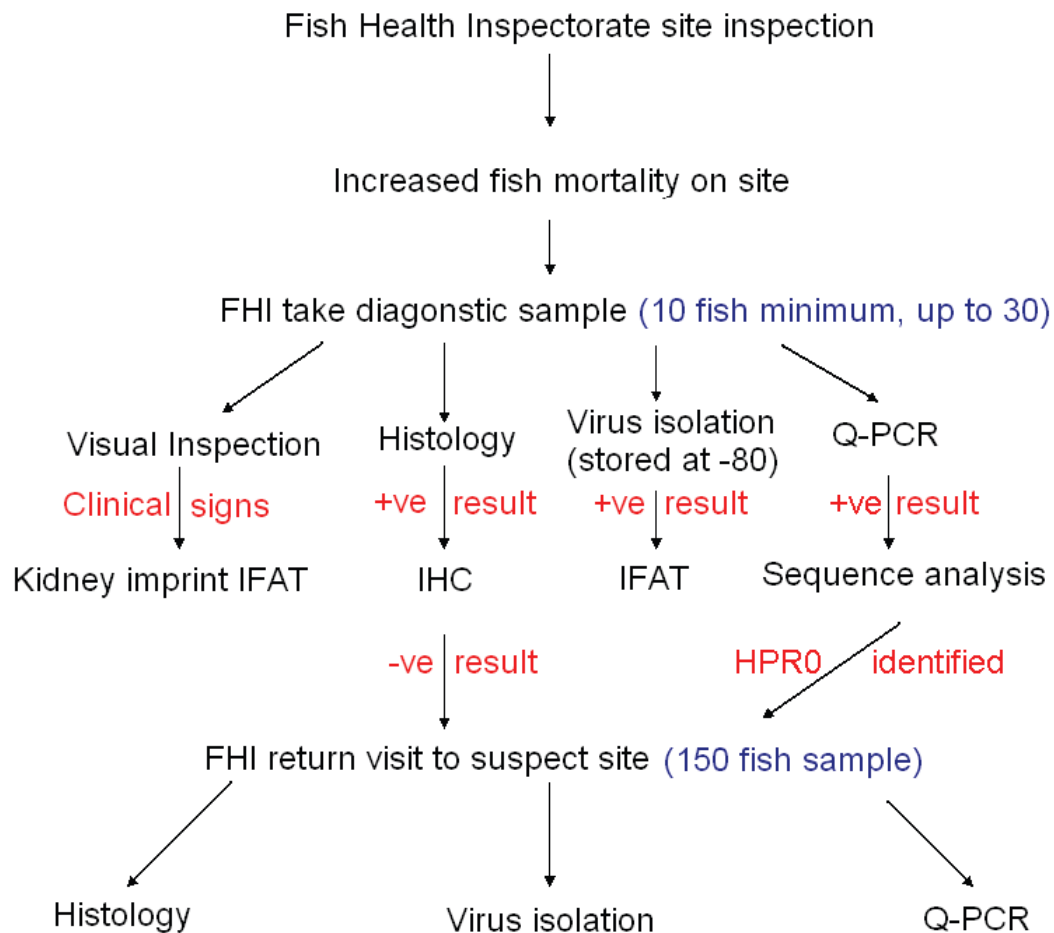


Figure 1 Diagnostic test procedures for confirmation of ISA

Gross pathological and histopathological are changes that are detailed in the Manual of Diagnostic Tests for Aquatic Animals (OIE, 2009).

Agent Detection and Identification Methods

Indirect Fluorescent Antibody Test

An indirect fluorescent antibody test (IFAT) using a validated monoclonal antibody (MAb, 3H6F8; NVI, Norway) against ISAV haemagglutinin-esterase (HE) on kidney imprints is performed on a case where clinical signs consistent with ISA disease are observed by Marine Scotland fish health inspectors.

Preparations of Kidney Imprints

In the field, a section of mid-kidney is briefly blotted against absorbent paper to remove excess fluid and blood, and imprints blotted on poly-L-lysine-coated microscope slides. The imprints are air-dried, fixed in acetone/methanol (1:1 v/v) for 5 min and stored at 4°C for a maximum of 72h until use.

Staining Procedure

After blocking with 6% skimmed milk in phosphate-buffered saline (PBS) containing 0.2% tween 20 (PBSt) for 30 min, the samples are incubated for 1 h with a 1:100 dilution of anti-ISAV MAb (3H6F8) in 1% skimmed milk, followed by three washes. For the detection of bound antibodies, the imprinted slides are incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse Ig (Sigma-Aldrich, UK) at a 1:1000 dilution in 1% skimmed milk for 1 h. The slides are again washed three times before incubation with CITIFLUOR AF1 solution (CITIFLUOR Ltd., UK) at a (1:4 v/v dilution in PBS). A further three washes are performed and the slides counter stained with propidium iodide (0.01mg/ml) for 3 min. The slides are once again washed 3 times and a coverslip placed on the slides aided by VECTASHIELD® Hard-Set™ (Vector Laboratories Inc., USA) mounting medium. PBS with 0.1% Tween 20 is used for all washing steps, all incubations are performed at room temperature and the microscope slides are kept in the dark until reading commences. Slides are read using an Olympus BX60 upright fluorescent microscope (Olympus America Inc, USA).

Immunohistochemistry

Tissue material for histological examination is sampled in the field and returned to the Marine Laboratory for processing. Paraffin sections from formalin-fixed tissues are prepared according to standard histological methods. Slides containing gill; gut; heart; kidney; liver; spleen, pancreas and skin tissue, along with confirmed positive and negative control slides are prepared for immunohistochemistry (IHC), where a monoclonal antibody against the ISAV nucleoprotein (MAb P10; Aquatic diagnostics Ltd., UK) is used to detect the presence of the virus within the tissue. Stained slides

are subsequently analysed by microscopy and the presence of ISAV is indicated by red staining of the tissue.

Preparation of Tissue

The tissues (gill; gut; heart; kidney; liver; spleen, pancreas and skeletal muscle) are fixed in neutral phosphate-buffered 10% formalin for a minimum of 24h, dehydrated in graded ethanol, cleared in xylene and embedded in paraffin, according to standard histological protocols. Approximately 3 μm thick sections are cut onto poly-L-lysine-coated slides and heated at 45–50°C for a minimum of 12 h, de-waxed in clearene and rehydrated through graded ethanol.

Staining Procedure for IHC

All incubations are carried out at room temperature on a rocking platform, unless otherwise stated. A glass pen is used to circumscribe the sections on the glass slides, to allow better adherence of the ImmEdge pen (Vector Laboratories Inc., USA) once the slides are wet. Endogenous peroxidase activity within the tissues is blocked using 3% hydrogen peroxide solution in TBS for 5 min. Slides are washed once in Tris buffer saline (TBS) and once in TBS with 0.1% Tween 20 (TBSt). The slides are then blocked using 1% horse serum from VECTASTAIN® Elite ABC kit (Universal) (Vector Laboratories Inc., USA) to prevent non specific binding of the MAb. Excess liquid is removed and the slides are incubated with the anti-ISAV MAb (P10) at a 20 μgml^{-1} solution in TBS for 1h. The slides are washed three times in TBSt and incubated with a secondary biotinylated horse antibody solution from the ABC kit for 30 min as per manufacturer instruction. The slides are once again washed in TBSt and covered with a peroxidise Avidin-Biotin Complex (ABC) reagent for 30 min. After this step, the slides are washed twice in TBSt and once with TBS and incubated with AEC staining solution (Sigma-Aldrich, UK) for 10min to detect the presence of peroxidise within the ABC/antibody bound complex. The slides are placed in distilled water for 5min and counterstained with Haematoxylin QS (Vector Laboratories Inc., USA) for 2min with excess stain removed under running tap water. Coverslips are placed onto the slides using CITIFLUOR mounting medium (CITIFLUOR Ltd., UK). Before reading, the slides are left for a minimum of 1 h to allow the blueing process to occur. All slides are read using an Olympus BX60 upright microscope under brightfield conditions.

Agent Isolation and Identification

Cell Culture Isolation

Inoculation of cell monolayers

Approximately 1g of mixed tissue sample (kidney, liver, spleen, heart) is aseptically dissected into 9 mL of viral transport media (Liebovitz L-15 cell culture media supplemented with 10% newborn calf serum, gentamicin (50mg/ml), polymixin 'B' (10,000U/ml); pH 7.4 – 7.8) in the field by Marine Scotland fish health inspectors and transported chilled to the Marine Laboratory. The VTM solution containing the tissue samples are homogenised within 48 h of sampling using an Enfer ETDS VIII tissue homogeniser (Design Village, UK), clarified by centrifugation and the supernatant sterile filtered before being mixed at a 1:1 v/v ratio with infectious pancreatic necrosis virus neutralising polyclonal antiserum and incubated at 15°C for 1-2 h. The sample/IPNV antisera solutions are inoculated at a 1:1000 dilution (final dilution of tissue material to culture medium) onto 12 well plates containing a monolayer of TO cells (Wergeland H., Department of Fisheries and Marine Biology, University of Bergen). The TO cell monolayers are at a confluency of 60 – 80%, 24 – 48 h old and incubated at 15°C after inoculation. A negative cell control and a 6 serial dilution positive control (10^{-3} – 10^{-8} dilution series), using the Scottish ISAV reference isolate (390/98) are prepared on the days of sample inoculation and subcultivation.

Monitoring Incubation

Growth of the virus may lead to a cytopathic effect (CPE) which is observed microscopically. Inoculated TO cultures are examined every 7 days for the appearance of CPE. If CPE occurs, an aliquot of the cell culture medium is screened for ISAV by IFAT. If IPNV is identified as the causative agent of the CPE, fresh TO cultures are inoculated with tissue homogenate supernatant (previously stored at -80 °C) incubated with a lower dilution of IPNV antisera. If no CPE has developed after 14 days, the cell monolayers are screened by haemadsorption and subcultivated to fresh TO cultures.

Subcultivation Procedure

Aliquots of culture media from the primary culture plates are inoculated onto 12 well plates containing fresh TO cells (culture conditions as above) at a 1:10 dilution 14 days (or earlier when obvious CPE appears) after inoculation. The secondary cultures are incubated at 15 °C for 14 days and CPE monitored as detailed for primary cultures. At the end of the incubation period, or earlier if obvious CPE appears, the medium is collected for virus identification (IFAT). All primary and secondary cultures, including those that demonstrate no CPE and all positive and

negative control wells, are examined for the presence of ISAV by haemadsorption after the 14 day incubation period (or earlier when obvious CPE appears).

Haemadsorption Assay

In the absence of CPE, a haemadsorption test is performed to detect intra-cellular virus. Atlantic salmon erythrocytes are overlaid on the drained cell monolayer. If the TO cells are virus-infected the erythrocytes will adhere to the cell monolayer in discrete foci or haemadsorption centres. This test gives a presumptive identification of a fish myxovirus. Briefly, blood from Atlantic salmon is freshly collected from the Marine Laboratory stock aquaria and centrifuged to pellet the erythrocytes. The red blood cells are then diluted in cell culture media at a 1:2000 dilution. The cell culture media is removed from each of the sample wells, 0.5 mL of the erythrocyte/media suspension is gently layer onto the TO cell monolayer and the cultures are incubated at room temperature for 45 min. After this incubation period, the erythrocyte/media suspension is discarded and the cell monolayers washed three times in cell culture media prior to the cultures being read under an inverted microscope. ISA virus from a positive haemadsorption sample is identified by an immunofluorescent antibody test (IFAT).

Immunofluorescent Antibody Test

TO cell monolayers are freshly prepared in 24 well plates at a confluency of 60 – 80%, 24 – 48h old. TO cell monolayers are inoculated in duplicate with sample media supernatant at 1:5 and 1:25 dilutions and incubated at 15°C for 4-7 days. Duplicate negative cell controls are included on each sample and positive control plates. Positive control plates are set up as described above but using the neat ISAV Scottish reference strain 390/98.

Once CPE occurs on the TO monolayer, the cell culture media is removed and the cell monolayer rinsed once with PBS and fixed in 80% acetone for 20 min. The acetone is then discarded and the plates left to air dry for 1 h. The fixed cell monolayers are then incubated at 37°C for 30 min with anti- ISAV MAb (MAb mix; MAb 3H6F8 + 10C9F5, NVI, Norway) at either a 1:20 or 1:50 dilution in PBS. The anti- ISAV MAb is then removed and the culture is rinsed three times with PBS/0.05% Tween 20 (PBSt). A secondary FITC-conjugated goat anti-mouse (Ig) antibody (Sigma-Aldrich, UK), diluted 1:100 in PBS is applied to the fixed monolayer and incubated at 37°C for 30 min. The secondary antibody is removed and the monolayer rinsed a further three times with PBSt. TO cultures are examined using a Nikon diaphot UV inverted microscope (Nikon Corp, Japan).

Real-Time Reverse-Transcription Polymerase Chain Reaction

RNA Extraction

Kidney material (approximately 0.25g) for molecular examination is sampled into 1 mL of RNALater® (Sigma-Aldrich, UK) in the field and returned chilled to the Marine Laboratory for processing. Samples are homogenised using a tissue lyser (Qiagen, UK). Total RNA from kidney tissue (5 mg) is extracted using the fully automated BioRobot M48 (Qiagen, UK) which elutes 100µL of total RNA in a pre-defined volume of elution buffer. Negative extraction controls were conducted by performing a blank extraction. Controls were taken through subsequent RT and PCR steps.

Reverse Transcription

cDNA from the extracted RNA and the negative extraction control was generated using ABI Taqman® Reverse Transcription Reagents kit with random hexamers (Applied Biosystems, UK). Approximately 1.0µg total RNA in a volume of 7.7µl is added to 12.3 µl of the reverse transcriptase mixture, giving a final volume of 20µl containing 1x RT buffer (25mM Tris-HCl, pH 8.3, 37.5mM KCl), 5.5mM MgCl₂, 0.5mM of each deoxynucleoside triphosphate (dNTP), 1.25uM random hexamers, 0.4U RNase Inhibitor and 1.25 U multiscribe reverse transcriptase. The mixture is incubated for 10 min at 25°C, 30 min at 48°C, heat inactivated at 95°C for 5 min. A negative RT control reaction is performed replacing the RNA with RNase free-water and taken through subsequent PCR step. A positive control is also included in the assay, which can be distinguished from sample material in the Real-time PCR.

Real-Time PCR

RT-PCR primer pairs and Taqman® MGB probes (Applied Biosystems, UK) targeting ISAV segment 8 and the Atlantic salmon elongation factor 1 alpha reference gene (ELF1-α) are used for the diagnostic real-time assay. Both primers and probes for ISAV are targeted to conserved gene regions to ensure detection of all documented variants of ISAV. All probes were designed over an intron-exon splicing site to exclude amplification from potential genomic contamination. In a diagnostic context, the ELF 1- α controls, provide a powerful quality control of individual samples. Assaying for constitutively expressed endogenous genes also allows for quantification of pathogen nucleic acid levels and facilitates direct comparison of relative levels of pathogen detected between samples.

Quantitative PCR is performed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). The assays are carried out in a 20µl volume, containing 900nM of each primer, 250nM Taqman® probe, 1 x PCR SensiMix (dU), 2.5 mM MgCl₂, 1 x Uracil DNA Glycosylase (UNG) (Quantace), dH₂O and 1µl cDNA. The following universal cycling conditions are applied to ISAV diagnostic samples:-

Step	Temp	Time	Number of Cycles
1	37°C	10 Minutes	1 Cycle
2	95°C	10 Minutes	1 Cycle
3	95°C	15 Seconds	45Cycles
	60°C	1 Minute	

No-target controls (NTCs) were performed for each primer and probe set. The primers and probes utilised in this assay are detailed below.

Assay Name	Forward Primer	Reverse Primer	Probe	Positive Control Probe
ISAVS EG8	ISAVSEG8UNIF 5'- CTACACAGC AGGATGCAG ATGT-3	ISAVSEG8UNIR 5'-CAGGATGCCGG AAGTCGAT-3'	ISAVSEG8UNIM 2 6-FAM- CATCGTCGCT GCAGTTC-MGB	ARTIFICIAL PROBE 6-VIC- ACCGTCTAGC ATCCAGT- MGB
ELF RNA Salmon	EL.1A- ELAFSALM-F 5'- CCCCTCCAGGA CGTTTACAAA-3'	EL.1A-ELARSALM- R 5'- CACACGGCCAC AGGTACA-3'	EL.1A-ELAM1 6-FAM- ATCGGTGGTA TTGGAAC-MGB	N/A

Appendix 2 Complete Testing Histories of Confirmed Sites

Two tables are presented that include all diagnostic test results obtained from all samples taken by FHI from within MA 3a (Table 1) and from outside MA 3a but within Shetland (Table 2).

Appendix 2 Table 1

Complete testing history for sites in MA 3a from 9/12/08 to following on 26/11/2009

Site No	Site Name	Inspector	Case No	Case Type	Date Of Visit	Frequency	Result type
FS0673	East of Hildasay	Danny Pendrey	20080974	Diagnostic	09/12/2008	1/1	ISAP
FS0673	East of Hildasay	Danny Pendrey	20080982	Statutory sample (eg 150-fish test)	16/12/2008	8/30	KPCR
FS0673	East of Hildasay	Danny Pendrey	20080974	Diagnostic	09/12/2008	1/1	ISAH
FS0673	East of Hildasay	Danny Pendrey	20080982	Statutory sample (eg 150-fish test)	16/12/2008	7/30	ISAV
FS0936	Foreholm	Paul McKay	20090063	Statutory sample (eg 150-fish test)	06/01/2009	0/30	KPCR
FS0936	Foreholm	Paul McKay	20090063	Diagnostic	06/01/2009	0/30	ISAV
FS0515	North Papa	Paul McKay	20090064	Diagnostic	07/01/2009	0/1	KPCR
FS0447	Papa	Paul McKay	20090065	Diagnostic	07/01/2009	0/2	KPCR
FS0935	Flotta	Yvonne McMurchie	20090043	Diagnostic	07/01/2009	0/2	KPCR
FS0316	Setter Voe	Ron Smith	20090124	Diagnostic	07/01/2009	1/2	KPCR
FS0316	Setter Voe	Ron Smith	20090124	Diagnostic	07/01/2009	0/2	ISAV
FS0691	Sound of Hoy	Yvonne McMurchie	20090044	Diagnostic	08/01/2009	0/2	KPCR
FS0937	West of Burwick	Paul McKay	20090067	Diagnostic	08/01/2009	0/1	KPCR
FS0785	Spoose Holm	Ron Smith	20090138	Diagnostic	09/01/2009	0/1	KPCR
FS0447	Papa	Katy Urquhart	20090364	Statutory sample (eg 150-fish test)	12/01/2009	4/30	KPCR
FS0433	Langa Isle (East)	Danny Pendrey	20090365	Statutory sample (eg 150-fish test)	12/01/2009	0/30	KPCR
FS0447	Papa	Katy Urquhart	20090364	Statutory sample (eg 150-fish test)	12/01/2009	8/30	ISAV
FS0316	Setter Voe	Katy Urquhart	20090376	Statutory sample (eg 150-fish test)	13/01/2009	0/30	ISAP
FS0937	West of Burwick	Paul McKay	20090367	Statutory sample (eg 150-fish test)	13/01/2009	0/30	KPCR
FS0316	Setter Voe	Katy Urquhart	20090376	Statutory sample (eg 150-fish test)	13/01/2009	0/30	ISAV
FS0674	North Havra	Paul McKay	20090368	Statutory sample (eg 150-fish test)	14/01/2009	0/30	KPCR
FS0515	North Papa	Katy Urquhart	20090369	Statutory sample (eg 150-fish test)	14/01/2009	0/30	KPCR
FS0785	Spoose Holm	Katy Urquhart	20090366	Statutory sample (eg 150-fish test)	15/01/2009	0/5	KPCR
FS0785	Spoose Holm	Sonia Duguid	20090244	Statutory sample (eg 150-fish test)	16/01/2009	0/30	KPCR
FS0935	Flotta	Sonia Duguid	20090371	Statutory sample (eg 150-fish test)	17/01/2009	0/30	KPCR
FS0320	Loch of Strom	Danny Pendrey	20090164	Statutory sample (eg 150-fish test)	29/01/2009	0/30	KPCR
FS0548	Lea Trondra (East of Trondra)	Yvonne McMurchie	20090048	Statutory sample (eg 150-fish test)	04/02/2009	0/30	ISAP
FS0548	Lea Trondra (East of Trondra)	Yvonne McMurchie	20090048	Statutory sample (eg 150-fish test)	04/02/2009	0/30	ISAV
FS0433	Langa Isle (East)	Paul McKay	20090075	Diagnostic	10/02/2009	0/1	KPCR
FS0433	Langa Isle (East)	Paul McKay	20090075	Diagnostic	10/02/2009	0/1	ISAV
FS0447	Papa	Paul McKay	20090081	Diagnostic	11/02/2009	1/3	KPCR
FS0447	Papa	Paul McKay	20090081	Diagnostic	11/02/2009	2/2	ISAI
FS0447	Papa	Paul McKay	20090081	Diagnostic	11/02/2009	1/3	ISAV
FS0447	Papa	Danny Pendrey	20090178	Research (eg FCB aquarium)	05/03/2009	0/10	KPCR
FS0447	Papa	Danny Pendrey	20090178	Research (eg FCB aquarium)	05/03/2009	0/10	GPCR
FS0447	Papa	Danny Pendrey	20090178	Research (eg FCB aquarium)	05/03/2009	0/60	ISAV
FS0515	North Papa	Danny Pendrey	20090180	Diagnostic	11/03/2009	2/2	ISAP
FS0515	North Papa	Danny Pendrey	20090180	Diagnostic	11/03/2009	2/3	ISAH
FS0515	North Papa	Danny Pendrey	20090180	Diagnostic	11/03/2009	2/2	ISAV
FS0515	North Papa	Danny Pendrey	20090184	Diagnostic	18/03/2009	1/2	ISAP
FS0515	North Papa	Danny Pendrey	20090184	Statutory sample (eg 150-fish test)	18/03/2009	16/30	ISAP
FS0515	North Papa	Danny Pendrey	20090184	Diagnostic	18/03/2009	12/12	ISAI
FS0515	North Papa	Danny Pendrey	20090184	Statutory sample (eg 150-fish test)	18/03/2009	15/30	ISAV
FS0433	Langa Isle (East)	Paul McKay	20090508	Diagnostic	14/05/2009	2/2	ISAI
FS0433	Langa Isle (East)	Paul McKay	20090508	Diagnostic	14/05/2009	1/1	KPCR
FS0433	Langa Isle (East)	Paul McKay	20090508	Diagnostic	14/05/2009	5/5	ISAA
FS0433	Langa Isle (East)	Paul McKay	20090508	Diagnostic	14/05/2009	1/1	ISAV
FS0548	Lea Trondra (East of Trondra)	Danny Pendrey	20090198	Diagnostic	19/05/2009	3/3	ISAP
FS0548	Lea Trondra (East of Trondra)	Danny Pendrey	20090198	Statutory sample (eg 150-fish test)	19/05/2009	25/30	ISAP
FS0548	Lea Trondra (East of Trondra)	Danny Pendrey	20090198	Diagnostic	19/05/2009	6/12	ISAI
FS0548	Lea Trondra (East of Trondra)	Danny Pendrey	20090198	Diagnostic	19/05/2009	12/12	ISAA
FS0548	Lea Trondra (East of Trondra)	Danny Pendrey	20090198	Diagnostic	19/05/2009	3/3	ISAV
FS0548	Lea Trondra (East of Trondra)	Danny Pendrey	20090198	Statutory sample (eg 150-fish test)	19/05/2009	27/30	ISAV
FS0316	Setter Voe	Paul McKay	20090961	Diagnostic	28/10/2009	1/1	ISAV
FS0316	Setter Voe	Paul McKay	20090961	Diagnostic	28/10/2009	1/1	ISAA
FS0316	Setter Voe	Paul McKay	20090961	Diagnostic	28/10/2009	1/1	KPCR
FS0316	Setter Voe	Sonia Duguid	20091021	Research (eg FCB aquarium)	09/11/2009	0/2	ISAV

Frequency (2nd last column) is the number of positive pools divided by the total number of pools in the sample. Result type (last column of each table) codes are: ISAA = ISA (histology); ISAH = ISA Immunohistochemistry (IHC); ISAG = ISA PCR (gill sample); ISAI = ISA (IFAT); ISAP = ISA PCR (kidney); ISAV = ISA virus isolation;

KPCR = ISA real time qPCR (kidney); GPCR = ISA real time qPCR (gill). These diagnostic tests are described in appendix 1.

Appendix 2 Table 2

Complete testing history for sites in Shetland outside MA3a during 2009. Note that all results are negative.

Site No	Site Name	Inspector	Case No	Case Type	Date Of Visit	Frequency	Result type
FS0666	Burrastow	Paul McKay	20090079	Diagnostic	28/01/2009	0/1	KPCR
FS0620	Aith (East)	Yvonne McMurchie	20090050	Statutory sample (eg 150-fish test)	02/02/2009	0/30	KPCR
FS0620	Aith (East)	Yvonne McMurchie	20090050	Statutory sample (eg 150-fish test)	02/02/2009	0/30	ISAV
FS1053	Hogan	Andrea Warwick	20090324	Statutory sample (eg 150-fish test)	03/02/2009	0/30	KPCR
FS1053	Hogan	Andrea Warwick	20090324	Statutory sample (eg 150-fish test)	03/02/2009	0/30	ISAV
FS0666	Burrastow	Andrea Warwick	20090325	Statutory sample (eg 150-fish test)	05/02/2009	0/30	KPCR
FS0397	Dales Voe	Yvonne McMurchie	20090049	Statutory sample (eg 150-fish test)	05/02/2009	0/30	KPCR
FS0397	Dales Voe	Yvonne McMurchie	20090049	Statutory sample (eg 150-fish test)	05/02/2009	0/30	ISAV
FS0666	Burrastow	Andrea Warwick	20090325	Statutory sample (eg 150-fish test)	05/02/2009	0/30	ISAV
FS0397	Dales Voe	Danny Pendrey	20090421	Diagnostic	06/04/2009	0/1	ISAP
FS0397	Dales Voe	Danny Pendrey	20090421	Diagnostic	06/04/2009	0/1	ISAV
FS0501	Taing of Railsbrough Catfirth	Danny Pendrey	20090431	Diagnostic	08/04/2009	0/1	ISAV
FS0501	Taing of Railsbrough Catfirth	Danny Pendrey	20090431	Diagnostic	08/04/2009	0/1	ISAP
FS0608	Vidlin North	Danny Pendrey	20090191	Diagnostic	29/04/2009	0/1	ISAP
FS1043	Ness of Copister	Paul McKay	20090509	Diagnostic	25/05/2009	0/1	KPCR
FS1043	Ness of Copister	Paul McKay	20090509	Diagnostic	25/05/2009	0/1	ISAV
FS0088	Cloudin	Andrea Warwick	20090633	Diagnostic	15/06/2009	0/2	KPCR
FS0088	Cloudin	Andrea Warwick	20090633	Diagnostic	15/06/2009	0/2	ISAV
FS0814	Swarta Skerry	Sonia Duguid	20090569	Diagnostic	07/07/2009	0/1	KPCR
FS0814	Swarta Skerry	Sonia Duguid	20090569	Diagnostic	07/07/2009	0/1	ISAV
FS0079	Brindister	Katy Urquhart	20090686	Diagnostic	05/08/2009	0/1	KPCR
FS0715	Linga	Katy Urquhart	20090685	Diagnostic	05/08/2009	0/2	GPCR
FS0715	Linga	Katy Urquhart	20090685	Diagnostic	05/08/2009	0/2	ISAV
FS0079	Brindister	Katy Urquhart	20090686	Diagnostic	05/08/2009	0/1	ISAV
FS1053	Hogan	Danny Pendrey	20090782	Diagnostic	18/08/2009	0/1	KPCR
FS0166	Holm of Gruting	Danny Pendrey	20090783	Diagnostic	18/08/2009	0/1	KPCR
FS0167	Mid Taing	Danny Pendrey	20090784	Diagnostic	18/08/2009	0/1	KPCR
FS0167	Mid Taing	Danny Pendrey	20090784	Diagnostic	18/08/2009	0/1	ISAV
FS0166	Holm of Gruting	Danny Pendrey	20090783	Diagnostic	18/08/2009	0/1	ISAV
FS1053	Hogan	Danny Pendrey	20090782	Diagnostic	18/08/2009	0/1	ISAV
FS0715	Linga	Sonia Duguid	20090577	Diagnostic	24/08/2009	0/1	KPCR
FS0079	Brindister	Sonia Duguid	20090578	Diagnostic	24/08/2009	0/1	KPCR
FS0079	Brindister	Sonia Duguid	20090578	Diagnostic	24/08/2009	0/1	ISAV
FS0715	Linga	Sonia Duguid	20090577	Diagnostic	24/08/2009	0/1	ISAV
FS0408	Poseidon	Danny Pendrey	20090920	Diagnostic	01/10/2009	0/2	KPCR
FS0408	Poseidon	Danny Pendrey	20090920	Diagnostic	01/10/2009	0/2	ISAV
FS1060	Wick of Garth	Mark Paterson	20090878	Diagnostic	15/10/2009	0/1	KPCR
FS1060	Wick of Garth	Mark Paterson	20090878	Diagnostic	15/10/2009	0/1	ISAV
FS0397	Dales Voe	Paul McKay	20090960	Diagnostic	27/10/2009	0/1	KPCR
FS0183	South Sound	Paul McKay	20090958	Diagnostic	27/10/2009	0/1	KPCR
FS0183	South Sound	Paul McKay	20090958	Diagnostic	27/10/2009	0/1	ISAA
FS0397	Dales Voe	Paul McKay	20090960	Diagnostic	27/10/2009	0/1	ISAV

Appendix 3 Procedure for Notification of Affected Companies

ISA is a notifiable disease in Scotland, as such a representative of fish farm companies, veterinarians or any other individual who has reason to suspect the presence of ISA on a site is legally obliged to report this suspicion to Scottish Ministers. The relevant legislation is now Council Directive 2006/88/EC which amalgamated and updated the relevant fish health legislation as interpreted under The Aquatic Animal health (Scotland) Regulations 2009. However, prior to March 2009, The Diseases of Fish Acts 1937 and 1983, EC Directives 91/67/EEC and amendments e.g. CD 2003/466/EC were in force. Whilst the name of the movement restrictions imposed under this legislation was changed from DAOs and TDNs to Initial Designation Notices (IDN) and Confirmed Designation Notices (CDN), the controls imposed remained similar except that the TDO and DAO did not control the movement of dead fish or equipment, being reliant of Control Notices for dead fish and Gate Notices for equipment.

Once informed of a suspicion, or as a result of their own routine surveillance, the FHI are required to investigate by site inspection investigating by diagnostic sampling and, where applicable, taking statutory samples. These are taken as soon as logistically possible following notification; realistically this requires 2-3 working days.

The company representative, as registered with Marine Scotland Science for disease control purposes, is immediately informed on receipt of any result from the diagnostic laboratory, by FHI, initially by phone but with a follow up written report.

In the case of a non-definitive result (insufficient to confirm or rule out ISA), as occurred initially at site A, a Thirty Day Notice (TDN) was served on the site under 4A of the Diseases of Fish Act 1937, as amended. This allowed official suspicion and confirmatory testing to be completed.

Where a single diagnostic test, such as PCR is unsupported by further test results (see Table 1 in main text), the criteria for official suspicion will not be met and movement restrictions will be lifted from that site on completion of the case. Where a site is under official suspicion, it remains suspect until six months have passed, in the absence of further evidence leading to confirmation.

When further laboratory results became available (on 2 January 2009 in the case of this outbreak) the presence of ISA was confirmed. Companies in the affected area were informed by phone, fax and email; however as this was a national holiday not all companies responded to contacts. Backup phone message were left, combined with emails. All companies were informed as a priority and as soon as practically possible.

A Designated Area Orders (DAO), prepared under the terms of section 2 of the Diseases of Fish Act 1937, as amended, were prepared for every fish farming site in MA 3a and faxed to the business correspondent of the companies concerned. Movement restrictions are placed in the form of: a Confirmed Designation Notice (CDN), formally Designated Area Order (DAO) which controls the movement of live fish and food and removal of mortalities; a Gate Notice (GN) which controls movement of staff, equipment and dead fish, or a Control Notice (CN) which controls movement of dead fish, are placed on sites within Control and Surveillance Zones. SGMS issue written notification to each fish farm company within the control and surveillance zones, detailing movement restrictions on each site.

During the course of the outbreak, subsequent results from tests undertaken on fish farm sites in MA 3a were communicated to company correspondents by telephone, and followed up with the issue of a written fish health report, following agreed Fish Health Inspectorate (FHI) procedures.

Where positive results were obtained from biological or chemical diagnostic testing the case was reviewed by senior diagnosticians and scientists to consider whether there was sufficient evidence to confirm or officially suspect the presence of ISA (see table 1 in main text). The criteria considered are defined by the OIE and also laid down by the European Commission in Commission Decision 2003/466/EC for the suspicion of infection with ISA or confirmation of ISA. The purpose of these reviews was to ensure that the results of the laboratory results were valid and to measure whether the criteria for suspecting or confirming the presence of ISA had been met.

Where the presence of ISA was confirmed, this information was passed to Scottish Government who, on their agreement with the MSS diagnosis, issued a withdrawal notice and was also communicated to the business correspondent of the relevant company. Scottish Government then issued a withdrawal notice, initially in accordance with The Diseases of Fish (Control) Regulations 1994, subsequently under The Aquatic Animal health (Scotland) Regulations 2009. The MSS decision and the withdrawal notice were communicated to the business correspondent of the affected company.

During the outbreak, concern was raised by industry representatives that insufficient information was being passed to affected companies with regard to the confirmation of ISA. Whilst the FHI were following current legislation and current and established policy practices additional information was also passed to any company operating a site where ISA was confirmed. This information included; the criteria for confirmation of the disease referencing both the OIE and any relevant legislation; the observations made by FHI on-site; the observations made by FHI at post-mortem and the results of any laboratory tests. This information was sent out by e-mail and followed with a hard copy by post on the day of confirmation.

The working practices of the FHI have now been updated, so in any future outbreak, this information will be supplied as a matter of course.

Appendix 4 Terms and acronyms used in this report

Terms and acronyms relating to the virus	
ISA	Infectious salmon anaemia disease
ISAV	Infectious salmon anaemia virus (not necessarily causing ISA)
HPR0	Putative ancestor ISAV, believed to be avirulent
2008-0982	Virus causing 2008/9 outbreak in Shetland
1998-390	Virus causing most of 1998/9 outbreak in Scotland
Terms and acronyms relating to diagnostic methods	
TO cell	A cell line originally cultured from salmon head kidney leucocytes used to replicate ISAV
IFAT	Indirect fluorescent antibody test (a diagnostic test)
qPCR	quantitative Real-Time Polymerase Chain Reaction (a diagnostic test)
Se	Test Sensitivity: probability that if infection is present test detect it
Sp	Test Specificity: probability that a negative sample will not test positive
Pooling	Taking tissues from multiple fish to reduce diagnostic costs
Other terms acronyms	
MSS	Marine Scotland Science
FHI	Fish Health Inspectors from MSS
MA	Management Area as defined by MSS
LPP	Lerwick Processing Plant processing plant
SPP	Scalloway Processing Plant
'bus stop'	The practice of collecting fish from multiple farms to take on to a processing plant or other destination
Synchronous fallowing	Fallowing all the sites in a defined area (ideally an MA) at the same time

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