

Detection of salmonid alphavirus RNA in Celtic and Irish Sea flatfish

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ABSTRACT: Pancreas disease (PD) caused by the salmonid alphavirus (SAV) has been the most significant cause of mortalities in Irish farmed salmon *Salmo salar* L. over the past decade. SAV is a single-strand positive-sense RNA virus, originally thought to be unique to salmonids, but has recently been detected using real-time RT-PCR in a number of wild non-salmonid fish. In the present report, 610 wild flatfish (common dab *Limanda limanda*, plaice *Pleuronectes platessa* and megrim *Lepidorhombus whiffiagonis*) were caught from the Irish and Celtic Seas and screened for SAV using real-time RT-PCR and sequencing. In general, a very low prevalence was recorded in common dab and plaice, except for 1 haul in Dublin Bay where 25% of common dab were SAV-positive. SAV sequence analysis supported the fact that real-time RT-PCR detections were specific and further characterised the detected viruses within SAV Subtype I, the predominant subtype found in farmed salmon in Ireland.

KEY WORDS: SAV · Pancreas disease · Wild fish · Horizontal transmission · Atlantic salmon · Aquaculture

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INTRODUCTION

Salmonid alphaviruses (SAVs) are recognised as serious pathogens of farmed Atlantic salmon (*Salmo salar* L.) and cause a pathology characterised by acinar pancreatic necrosis, with cardiac and skeletal myopathies known as pancreas disease (PD). PD usually affects the marine production cycle, where first-year smolts have a high risk of infection associated with horizontal spread (Jansen et al. 2010, Graham et al. 2011, Stene et al. 2013). In Ireland, annual mortality levels over the last decade have ranged between 14 and 18%, reaching 40% in badly affected cages. PD has been a problem in Ireland since the early 1990s and has since become a leading cause of mortality in farmed Atlantic salmon (Crockford et al. 1999, Rodger & Mitchell 2007). Recent classification of SAV using sequence data derived from the E2 surface glycoprotein gene has led to the recognition of 6 different virus subtypes. Representatives of these

subtypes, except Subtype III, have been isolated from either Scotland or Ireland (Fringuelli et al. 2008, Graham et al. 2012a). The role of infected fish in the spread of pancreas disease through horizontal transmission is well established (McLoughlin et al. 1996). However, there is still a lack of information on other possible vectors of virus transmission such as wild fish and sea lice. Graham et al. (2006) found virus-neutralizing antibodies in the serum of saithe *Pol-lachius virens* sampled from Atlantic salmon cages with SAV-infected fish. Using molecular methods, Petterson et al. (2009) detected SAV Subtype III in the sea louse *Lepeophtheirus salmonis* in Norway, while Snow et al. (2010) detected SAV RNA in kidney and heart tissues of flatfish off the coast of Scotland. The aim of this study was to determine the prevalence and subtype of SAV, if present, in flatfish, i.e. common dab *Limanda limanda*, plaice *Pleuronectes platessa* and megrim *Lepidorhombus whiffiagonis* sampled off the coast of Ireland.

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MATERIALS AND METHODS

Fish were sampled onboard research vessels demersal trawling in the Irish and Celtic Seas, using a 2 to 3 metre beam trawl. Fish were caught from 4 trawls in the Irish Sea: 3 in Dublin Bay (October 2010 twice and February 2011) and 1 in Wexford Harbour (October 2010). A much broader annual groundfish survey was completed on the RV 'Celtic Voyager' in the Celtic Sea in December 2012, whereby the fish were caught from multiple hauls covering the south east to the north west of Ireland. In all cases, sampling was completed onboard within 1 h of capture. Either heart or gill tissue was taken and preserved by storage in RNAlater[®] for testing in the laboratory. Pancreas and liver tissue were collected from Irish Sea flatfish into neutral-buffered formalin, and stained using haematoxylin and eosin according to standard methods for histopathological examination.

Total RNA was extracted from 610 flatfish from either ~15 mg heart or gill tissue samples using a Qiagen TissueLyser and a standard protocol from the Qiagen RNeasy[®] mini kit. Gill tissue was used in this study because SAV persists for a long time in this tissue (Graham et al. 2010). RNA was eluted in a 50 µl volume and a subsample treated with Qiagen RNase-Free DNase. Treated RNA was subsequently purified using a Qiagen RNeasy minElute kit and stored at -80°C. Real-time RT-PCR primers targeting the nsP1 gene (Hodneland & Endresen 2006) were used to test for the presence of SAV RNA using a 1-step real-time reverse transcription-polymerase chain reaction (RT-PCR) with Platinum[®] qRT-PCR Thermoscript[™] (Invitrogen) and an ABI 7500 thermal cycler (Applied Biosystems). A total real time RT-PCR reaction volume of 20 µl, containing 2 µl of sample RNA, 300 nM of each primer and 200 nM of probe was subjected to the following thermal profile: 50°C for 20 min and 95°C for 15 min; 40 cycles of 94°C for 15 s and 56°C for 60 s; followed by a final holding stage of 72°C for 4 min. A process control assay targeting the 12S ribosomal subunit RNA in flatfish was also developed to monitor the nucleic acid extraction process of each sample and rule out false negative test results. Extraction process control primers 12Sf (AGA ACC GAT AAC CCC CGT TAA) and 12Sr (GAC GGT GGT ATA TAG GCG GAT AA) and probe 12Sp (FAM-CCT CAC CCT CTC TTG-MGB-NFQ) were designed with Primer Express 3.0 (Applied Biosystems) from alignment of 12S rRNA sequences for plaice (GenBank acc. no.: AF542207);

flounder (acc. no.: AF488491) and megrim (acc. no.: AF517556) using DNASTAR Lasergene 10 MegAlign. All primers were supplied by Sigma-Aldrich[®] and Taqman probes[®] were supplied by Applied Biosystems.

Samples were tested in duplicate for SAV. Each PCR assay plate included a negative process control, SAV positive control and a negative control. Due to the large number of samples tested, an extraction process control and internal process control (IPC, Life Technologies) was run for 10% of the samples. Samples from this survey were deemed positive for SAV by real-time PCR providing that threshold cycle (Ct) values were generated in each of the duplicate reactions tested for each fish; and when control assay criteria (i.e. extraction and amplification processes) were acceptable. SAV real-time RT-PCR positive samples were subsequently amplified by conventional RT-PCR targeting a 516bp variable region of the E2 gene (Fringuelli et al. 2008) using the Qiagen[®] 1-step RT-PCR kit on a Bio-Rad DNA Engine[®] thermal cycler. The RT-PCR thermal profile consisted of 50°C for 30 min and 95°C for 15 min; followed by 45 cycles of 94°C for 15 s, 58°C for 30 s, and 72°C for 50 s; and a final elongation step at 72°C for 10 min. RT-PCR was conducted at a final reaction volume of 50 µl including 8 µl template RNA. E2 PCR amplicons were visualised following separation by electrophoresis on a 1% (w/v) agarose gel. Candidate positive amplicons were sent for purification and sequenced commercially (Sequiseve). The consensus sequence results (from positions 9408 to 9764 of the E2 gene taken from SAV isolate F93-125; AJ316244.1) were compared with a number of SAV sequences representative of each subtype grouping: Subtype I F93-125 (AJ316244); Subtype II F04-198(22) (EF-675590) and S49p (AJ316246); Subtype III F04-170(7) (EF675594) and F04-170(6) (EF675591); Subtype IV F91-115 (EF675563); Subtype V F06-41(54) (EF675570); and also compared with a number of SAV strains previously identified in Scottish and Irish salmonids: F07-02 (EF67571) and Marine Institute isolates F89-10 and F100-10. Multiple sequence alignments were performed by Clustal W analysis (Thompson et al. 1997). The evolutionary history was inferred using the neighbour-joining method (Saitou & Nei 1987), and evolutionary distances computed using the Kimura 2-parameter method (Kimura 1980). One thousand bootstrap replicates were performed for each analysis, and all phylogenetic analyses were conducted using MEGA v.5 (www.megasoftware.net) (Tamura et al. 2011).

RESULTS AND DISCUSSION

All real-time PCR assays achieved acceptable results for amplification and extraction controls, and no PCR or process contamination was experienced. Endogenous 12S rRNA extraction process control samples performed reliably for each flatfish species. Genomic DNA was removed prior to PCR, since the 12S rRNA primers and probe are not specific to RNA and can act effectively for both RNA and DNA extraction process controls. Real-time SAV positive samples were subsequently confirmed with the use of conventional RT-PCR and sequence analysis of the E2 gene, which is unrelated to the nsP1 gene used for the real-time RT-PCR screening. In addition, positive gill samples were repeated from extraction to PCR to further increase the confidence of reporting such results.

Real-time RT-PCR results from this survey are summarised in Table 1 and Fig. 1. Twelve fish, from a sample of 97 common dab, tested positive by real-time RT-PCR for SAV RNA in the Irish Sea. Almost all were exclusive to 2 trawls caught in Dublin Bay sampled during October 2010 when 10 of 40 fish were SAV-positive compared with 2 of 25 positive fish from a subsequent sample taken in February 2011. Ct values ranged from 24 to 36 from these sample batches, with the lowest Ct values found in gill tissue. Most of the Ct values generated were >30, with an average Ct value of ~31. No positive samples were detected in the fish sampled from Wexford Harbour in October 2010. It should be noted that real-time Ct values were found in 1 out of 2 tested replicates from 2 of the Wexford Harbour fish and a further 3 fish from Dublin Bay (October 2010 sample). In these

instances the Ct values were >33, and at the higher end of the range detected in this report. A total of 513 fish were sampled and tested for SAV from the December 2012 survey of the Celtic Sea. These comprised 66 common dab, 228 plaice, and 219 megrim. Six fish tested positive for SAV RNA (5 plaice and 1 common dab) and only 2 of these positives came from the same haul. Ct values ranged from 29 to 35.

12S rRNA extraction process control samples performed reliably for the relevant samples tested. Ct values ranged between 10 and 13 for common dab and plaice, and 15 and 20 for megrim. These may provide a suitable extraction process control for other studies using flatfish.

Real-time RT-PCR QnsP1 Ct values from this survey were similar to those previously detected in wild flatfish by Snow et al. (2010). As tissue was sampled using RNeasy[®] it was not possible to attempt to culture the virus. Despite the absence of isolated virus from this study, 70% of SAV real-time positive samples were reproducible when repeated from nucleic acid extraction to PCR. Furthermore, 50% of SAV positives were confirmed by conventional reverse transcription PCR and sequence analysis. However, all real-time RT-PCR sample positives were attempted by conventional RT-PCR, but only those samples with the lowest Ct values were successfully confirmed. In light of these SAV detections, it is reasonable to assume that the common dab caught in Dublin Bay have experienced SAV exposure within their population community. This is supported by the high SAV prevalence detected from the October sample, which was much higher when compared with SAV detections in Celtic Sea flatfish, and flatfish tested for SAV by Snow et al. (2010). Interest-

Table 1. Irish flatfish species, sample location and tissue type tested with real-time RT-PCR for detection of salmonid alpha-virus (SAV) (QnsP1 assay). RNA was extracted from individual fish and assayed in duplicate. Positive results were standardised by the presence of threshold cycle (Ct) values produced in both sample replicates, although some fish produced a single Ct value (data not shown). Only the mean from both replicate Ct values is presented. One common dab fish sampled from Dublin Bay in February 2011 was positive for SAV RNA in both heart and gill tissue

Sample species	Sample date	Location	Tissue	Positive fish (total samples)	Sample mean QnsP1 Ct values
Dab <i>Limanda limanda</i>	October 2010	Dublin Bay, Irish Sea	Gill	10 (40)	31, 33, 36, 28, 36, 31, 24, 32, 29, 32
	February 2011	Dublin Bay, Irish Sea	Gill, heart	2 (25)	Gill: 29, 32; Heart: 32
	October 2010	Wexford Harbour, Irish Sea	Gill	0 (32)	
	December 2012	Celtic Sea	Gill	1 (66)	29
Plaice <i>Pleuronectes platessa</i>	December 2012	Celtic Sea	Gill	5 (228)	29, 35, 32, 32, 31
Megrim <i>Lepidorhombus whiffiagonis</i>	December 2012	Celtic Sea	Gill	0 (219)	

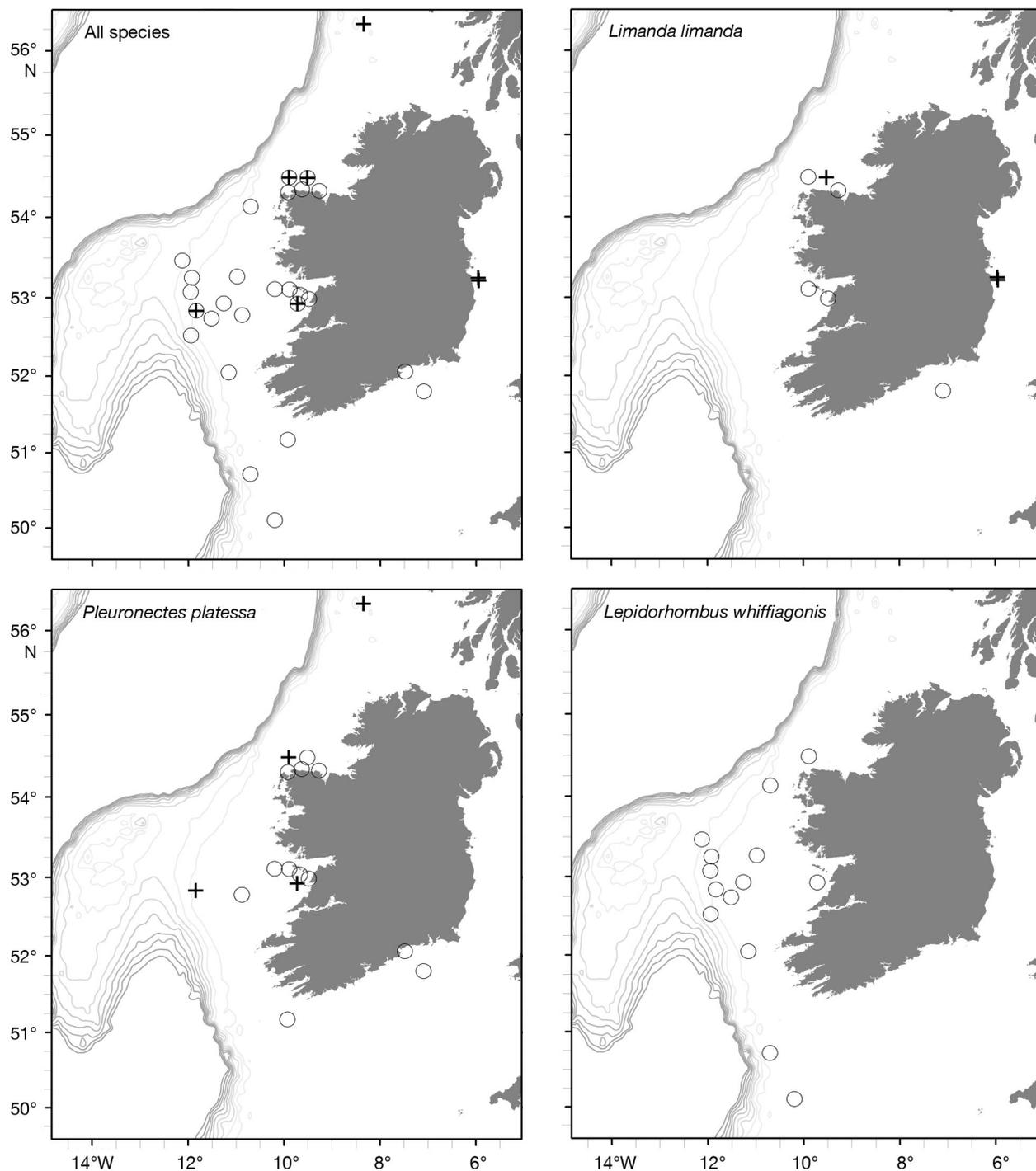


Fig. 1. Beam trawl sample locations tested using real-time PCR for the screening of salmonid alphavirus (SAV) in Irish and Celtic Sea flatfish. Location maps show results collectively for all species and individually for common dab *Limanda limanda*, plaice *Pleuronectes platessa*, and megrim *Lepidorhombus whiffiagonis*. (O) SAV-negative sample locations; (+) SAV-positive sample locations

ingly, similar prevalence and Ct values were reported in SAV outbreaks in farmed Atlantic salmon sampled at a comparable time of the year to the Dublin Bay common dab, using the same QnsP1 real-time

PCR testing methodology of gill tissue (Graham et al. 2010). Despite recording low Ct values and high prevalence for SAV in the Dublin Bay common dab, there was no evidence of typical PD histopathology

in pancreas and liver tissue sections. SAV detected in this survey were all characterised as Subtype I isolates, sharing 99% nucleotide identity with the SAV Subtype I reference strain (AJ316244.1) (Fig. 2). All 9 nucleotide sequences were highly similar, showing <0.6% differences. SAV Subtype I is the predominant subtype found during PD outbreaks in Atlantic salmon farmed in Ireland, and has remained largely unchanged over the last 10 years (Weston et al. 2005, Fringuelli et al. 2008, Graham et al. 2012a). Irish isolate F89-10 from farmed Atlantic salmon formed part of the same grouping as the SAV found in the wild flatfish, sharing $\geq 99.2\%$ nucleotide sequence identity. SAV Subtype IV is also found in farmed Atlantic salmon in Ireland (Fringuelli et al. 2008, Graham et al. 2012a) but was not found in this survey. Furthermore, SAV Subtype V found in wild flatfish species caught from the North Sea (Snow et al. 2010) was also absent from this survey. The same real-time RT-PCR and analysis of sequence region was used in all instances. Broad phylogenetic comparison of SAV has recently provided evidence of geographic clustering of subtypes (Graham et al. 2012a). Interest-

ingly, this was the first time that SAV subtype I has been detected in the Irish Sea and in wild flatfish species. This subtype is more commonly associated with farmed Atlantic salmon from the west coast of Ireland, therefore providing evidence that SAV Subtype I is more widespread around Ireland and not exclusive to the west coast aquaculture sites. Furthermore, due to the absence of nearby aquaculture facilities in the Irish Sea, the capture location of some of the SAV positive wild flatfish makes the route of exposure to SAV in these fish difficult to explain. Under experimental conditions, SAV has been detected in the water (Andersen et al. 2010), and Graham et al. (2012b) demonstrated that viable SAV is shed via the faecal route. These represent possible routes for horizontal SAV transmission to wild flatfish living in close proximity to aquaculture sites. The prevalence of other salmonid pathogens in wild fish, such as infectious pancreatic necrosis virus, is significantly higher within a 5 km radius of aquaculture sites around Scotland (Wallace et al. 2008). However, common dab found with SAV Subtype V captured off the NE coast of Scotland, and the common dab sam-

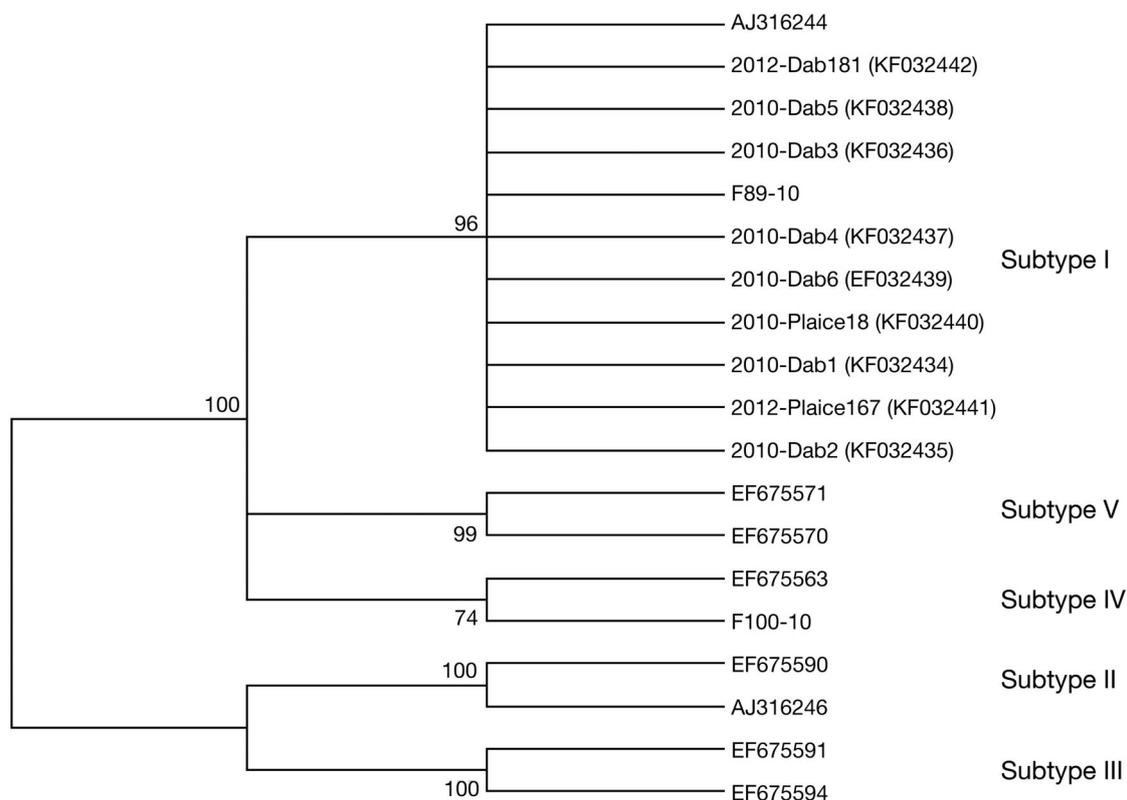


Fig. 2. Phylogenetic tree showing relationships between the 9 detected salmonid alphavirus (SAV) isolates from Irish flatfish and 2 isolates from farmed Atlantic salmon (F89-10, F100-10) with the main SAV subtypes according to a short nucleotide sequence (357 bp) comparison of the E2 gene (Fringuelli et al. 2008). The tree was constructed using the neighbour-joining method with 1000 bootstrap replicates using MEGA. Only branches with >70% support are shown

pled from Dublin Bay, were all remote from aquaculture facilities. Interestingly, the migration habits of Atlantic salmon and Irish Sea common dab, and also the diet analysis of Atlantic salmon, suggest that neither species is likely to interact in the wild (Jacobson & Hansen 2001, Haugland et al. 2006). Spares et al. (2007) and Dadswell et al. (2010) show that wild southern European (<62°N) salmonid stocks typically use the North Atlantic Sub-polar Gyre migration route, and Irish Atlantic salmon enter this predominantly from the west coast of Ireland. Unfortunately, comparatively little information exists for migration patterns and ecology of common dab. Irish Sea stock assessments by Fox et al. (1997) and Henderson (1998), and Irish Sea beam trawl survey data for common dab and plaice by Rogers et al. (1998) and Maxwell et al. (2009) suggest a relatively localised population habitat confined to coastal nurseries and offshore spawning grounds ≤30 to 50 m depth. Using this information as an inference of the habitat range for the Dublin Bay common dab, their distribution may be confined to the Dublin Bay region. Despite the uncertainties with common dab migration data, the fact also remains that Irish aquaculture sites operate on the west coast, from where 85% of the total flatfish survey samples were obtained with 99% testing SAV negative. However, SAV prevalence in Celtic Sea flatfish was based on sampling completed during the winter. Positive samples may therefore only reflect the most persistent detections (Graham et al. 2010). Detections from Dublin Bay could indicate potential SAV circulation in flatfish communities; however, seasonal effects on prevalence remain unknown. Collectively, the evidence from Snow et al. (2010) and from this report—most notably the SAV prevalence in the Irish Sea common dab as well as SAV detection in multiple tissues and in multispecies—seems to reinforce the suggestion of an alternative route of SAV exposure in farmed Atlantic salmon and a potential wild origin of SAV. Although more definitive evidence of SAV pathology in flatfish is necessary before one can consider SAV infection, epidemiological investigations have implicated wild fish as disease reservoirs for other salmonid pathogens (such as viral haemorrhagic septicaemia and infectious salmon anaemia viruses; Snow 2011). The plausibility of SAV in wild fish represents an interesting line of future research. Variability across the SAV genome to investigate host fish plasticity could lend an interesting insight toward this possibility. Also, it would be worthwhile concentrating efforts toward the culture of SAV from future Irish flatfish surveys. Furthermore, challenge experi-

ments to establish the nature and the potential infection risk posed by such carrier species could also provide a useful tool for disease management and risks associated with wild fish residing close to Atlantic salmon aquaculture sites.

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