

Sub-clinical infection of farmed Atlantic salmon *Salmo salar* with salmonid alphavirus— a prospective longitudinal study

D. A. Graham^{1,*}, H. Jewhurst², M. F. McLoughlin³, P. Sourd⁴, H. M. Rowley¹,
C. Taylor², D. Todd¹

¹Veterinary Sciences Division, Stoney Road, Stormont, Belfast BT4 3SD, UK

²Queen's University of Belfast, Stoney Road, Stormont, Belfast BT4 3SD, UK

³Aquatic Veterinary Services, 35 Cherryvalley Park, Belfast BT5 6PN, UK

⁴Fjord Seafood Scotland Ltd., 1 Marybank Industrial Estate, Stornoway, Isle of Lewis, HS2 0DB, UK

ABSTRACT: A prospective longitudinal study of salmonid alphavirus infection in farmed Atlantic salmon *Salmo salar* L. was initiated in post-transfer smolts on a UK farm in July 2004 and continued for 320 d. Sampling was concentrated on a single caged population (C4) with serum and tissue samples collected and tested for viraemia, virus neutralising (VN) antibodies and viral nucleic acid by real time RT-PCR and by histopathology; 380 sera collected between Days 0 (D0) and 139 (D139) were consistently negative for both viraemia and VN antibodies. The first evidence of infection was detected on D146, when 4 out of 20 fish were found to be viraemic and 1 of 20 to be antibody-positive. On D153 only 2 of 20 fish was viraemic and 1 antibody positive. At the next sampling (D158) no viraemic or antibody positive fish were detected. Thereafter, one or two viraemic fish were detected on 6 occasions, including on D320. The prevalence of antibody-positive fish remained low (0 to 5%) until D192 after which time it rose irregularly to a peak of 57.9% on D320. Real time RT-PCR testing of sera was more sensitive than screening for viraemia, detecting a peak of 35% positive on D153 before declining. Histological lesions diagnostic for pancreas disease (PD) were observed at D146 and D153 only. In addition, mild cardiac and to a lesser extent brain lesions were frequently found after virus was detected, but not in earlier samples. No clinical signs or mortalities attributable to PD occurred throughout the study. This is the first detailed report of sub-clinical infection and highlights the usefulness of longitudinal surveys and the detection of virus and antibodies as diagnostic and epidemiological tools.

KEY WORDS: Salmonid alphavirus · Pancreas disease · Longitudinal survey · Sub-clinical infection · Viraemia · Serology · RT-PCR

—Resale or republication not permitted without written consent of the publisher—

INTRODUCTION

Pancreas disease (PD) of farmed Atlantic salmon *Salmo salar* L. was first described in Scotland in 1976 (Munro et al. 1984). Since then it has also been described in North America (Kent & Elston 1987), Ireland (Murphy et al. 1992) and Norway (Poppe & Breck 1989). The condition is now known to be caused by infection with an alphavirus that is closely related to

the virus responsible for sleeping disease (SD) in farmed rainbow trout *Oncorhynchus mykiss* (Nelson et al. 1995, McLoughlin et al. 1996, 2002, Castric et al. 1997, Weston et al. 1999, Villoing et al. 2000). The name salmonid alphavirus (SAV) has been proposed for this closely related group of viruses (Weston et al. 2002).

PD has the potential to have a very important economic impact on farmed salmon production. Epidemi-

*Email: david.graham@afbini.gov.uk

ological studies in Ireland between 1989 and 1994 found that mortality levels on sites varied widely from a low of 0.1% to as high as 63% (Crockford et al. 1999). However, overall there was a significant downward trend in PD-related mortalities during this period that continued in the second half of the 1990s. A similar observation was reported from Scotland (Bruno 2004). However, in recent years PD has re-emerged as a significant cause of mortality and economic loss in the Irish industry (McLoughlin et al. 2003) and there is evidence of a similar trend in both Scotland and Norway (G Ritchie pers. comm.).

Both field and experimental infections of salmon with SAV isolates from outbreaks of PD are characterised clinically by sequential histopathological changes that include pancreatic and cardiomyocytic necrosis and skeletal myopathy (McLoughlin et al. 1996, 2002). Because of the difficulties inherent in isolating the virus from tissues of affected fish (Nelson et al. 1995), traditionally, these histopathological changes have been the basis on which PD is diagnosed. More recently, tests capable of screening large numbers of sera for the presence of both SAV viraemia and virus neutralising antibodies have been developed and evaluated for routine diagnostic and epidemiological use (Graham et al. 2003, 2005, Jewhurst et al. 2004).

Many different environmental, host and pathogen-specific factors have the potential to influence the severity of an outbreak of PD in a given population and the interrelationship between these is not yet clearly understood. In order to study the development of disease outbreaks, we previously published the first description of the sequential virological, serological and histopathological changes that occur during the course of clinical outbreaks (Graham et al. 2005). However, the sampling intervals in that study were irregular and weeks to months apart. In order to gain a better understanding of the epidemiology of a natural outbreak of PD, a prospective longitudinal study with regular, frequent sampling was conducted over an 11 mo period. This paper describes the outcome of this study.

MATERIALS AND METHODS

Farm details and collection of samples. Samples were collected from a UK salmon farm which had lost approximately 30% of fish in the previous production cycle to PD. Sampling was concentrated on a single population of S1 smolts in Cage 4 (C4), chosen by the farm veterinarian as being representative of the site. These fish had been transferred to the sea in April 2004, and had experienced an outbreak of infectious pancreatic necrosis (IPN) prior to the study period.

Sampling of C4 commenced on 20 July 2004 (Day 0, D0) and continued until June 2005. Sampling was performed at approximately weekly intervals initially, although in the second half of the study the inter-sample interval increased. At each sample point, blood samples were collected from 20 fish and sera separated by centrifugation. In addition, pancreas, heart, brain and red (RM) and white (WM) skeletal muscle samples were taken from the first 5 fish at each sampling and placed in 10% neutral buffered formalin for subsequent histopathological examination. For part of the study, heart tissue from these fish was also placed in *RNAlater*[®] (Ambion) for subsequent processing by real time RT-PCR (RRT-PCR). Sera were also collected from fish in other cages (5 to 20 cage⁻¹) on the site on a number of occasions. Data on water temperature, clinical signs of disease, lice numbers and mortalities were available from the farm records.

Histopathology. Tissues for histology were processed by standard paraffin wax techniques and stained with haematoxylin and eosin (H&E) for examination. The distribution and severity of tissue lesions, where present, were recorded as mild, moderate or severe.

Virological and serological testing. Virus neutralization (VN) testing and screening for viraemia were performed as previously described (Graham et al. 2003, Jewhurst et al. 2004). Briefly, sera were initially screened in duplicate at dilutions of 1/20 and 1/40 for virus neutralising (VN) antibodies in a microtitre-based test containing 30 to 300 TCID₅₀ of the reference salmon SAV strain (F93-125). The test was read after 3 d using a SAV-specific monoclonal antibody and an immunoperoxidase-based staining method (Welsh et al. 2000). Sera that neutralised virus in 1 or both wells at a dilution of 1/20 were considered positive. VN titres were calculated according to the method of Karber (1931). In parallel with the VN screen, sera were inoculated onto chinook salmon embryo-214 (CHSE) cells and stained after 3 d to identify viraemic sera.

RNA extraction and RRT-PCR. RNA extraction from sera and heart, and subsequent testing by RRT-PCR was conducted as previously described (Graham et al. 2006). Briefly, RNA was extracted from 200 µl of sera using the High Pure Viral RNA kit (Roche) and RNA extraction from heart tissue was performed using the QIAGEN RNeasy Mini Kit (Qiagen). One-step RRT-PCR was then performed using the Quantitect SYBR Green RT-PCR kit (Qiagen) with a primer pair designed to amplify a 227 bp product of the E1 region of SAV. All kits were used according to the manufacturers' instructions. Water samples (typically 1 for each 5 test samples) were extracted as negative controls to demonstrate the absence of cross-contamination during processing.

RESULTS

Clinical observations and management data

Serum and tissue samples were collected from Cage 4 on a total of 32 occasions over a 320 d period. During this period, serum samples were collected from other cages on a number of occasions and tested for viraemia and VN antibodies. Water temperatures on D0 were 12.5°C and peaked at 14.5°C in late August. Thereafter, temperatures declined to a low of 6.5°C in March 2005, before recovering to 9.5°C at the end of the study. Throughout the study period there were no clinical signs of PD and no significant mortalities attributed to PD. Available data indicated that between D13 and D320 weekly mortality rates for C4 were <0.05% except on 5 occasions between August 2004 and June 2005 when weekly mortalities were 0.1, 0.09, 0.18, 0.32 and 0.34%. The last 2 of these spikes were associated with grading. Fish on the site, including those in C4 were first graded using a well boat in November 2004. Subsequently, passive grading was carried out in C4 on D245, D280 and D320. On each occasion, the smaller fish were retained in C4. An increase in lice *Caligus* sp. numbers was observed in November 2004, which peaked with a count of 17 fish⁻¹ in December, before successful treatment.

Virology and serology

From July to early December 2004 (D139), a total of 19 sets of sera (n = 380) from C4 were tested and consistently found to be negative for detectable viraemia and VN antibodies. Viraemia was first detected at D146, when 4 (20%) of 20 sera were positive (Fig. 1). A single serum in this sample was also antibody positive (5%). At the next sampling (D153) the prevalence of viraemic fish declined to 10% and subsequently to 0% on D158. Thereafter, the detected prevalence of viraemia fluctuated between 11.8 and 0%, with a single viraemic fish (5%) being detected at the last sampling point (D320), 174 d after the first positive result. In total, 13 viraemic sera were detected. The prevalence of seropositive fish in C4 remained low (0 to 5.8%) from D146 to D183. Thereafter, the seroprevalence rose to 15% (D192) and continued to increase to 50% by D211. The seroprevalence in C4 was

variable thereafter, but a peak value of 57.9% was reached at the last sample point (D320, Fig. 1). Individual VN titres ranged from 1/20 to >1/40. Details of the results obtained with sera from other cages on the site at different time points are shown in Table 1. In the first cross-sectional sample taken on D153 individual cages showed different results, with some sero- and virus-negative, some viraemic but sero-negative, some viraemic and sero-positive and some virus-negative but sero-positive. Thereafter, the trend was for increasing seroprevalence and decreasing or absent viraemia. All of the viraemic samples detected were VN-negative. A single serum sample was also collected on D302 from a saithe *Pollachius virens* in the vicinity of Cage 6. This sample was found to be virus-negative but had a VN titre >1/40. To investigate this further, 3 additional sets of sera were collected on the site from saithe after the end of the study period on D337 (n = 4), D439 (n = 5) and D504 (n = 19). Two of the samples collected on D337 were toxic in cell culture and the remainder was seronegative. One sample

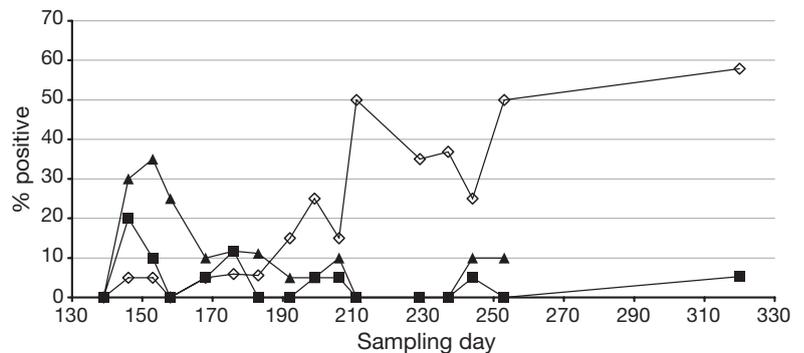


Fig. 1. *Salmo salar*. Percent sera positive for virus in cell culture (■) and RRT-PCR (▲) and for virus neutralising antibody (◊) at each sampling point from Day 139 onwards (all previous results were negative)

Table 1. *Salmo salar*. Details of virological and serological testing of serum taken from other cages at same site as Cage 4. Data are no. of fish tested/ no. viraemic/no. antibody-positive. -: not sampled

Cage No.	Day						
	153	163	192	198	244	272	302
1	5/1/0	5/0/2	10/0/7	-	-	-	-
2	10/1/1	-	10/0/8	-	-	-	-
3	5/0/0	-	-	-	-	-	-
5	5/0/1	-	-	-	-	-	-
6	10/0/1	-	-	-	-	20/0/13	20/0/9
8	10/1/0	-	-	10/0/6	10/0/7	-	-
9	5/0/1	-	-	-	-	-	-
10	10/0/0	-	-	-	-	-	-
11	5/1/1	-	-	-	-	-	10/0/10
12	5/0/0	-	-	-	-	-	-
13	5/0/4	-	-	-	-	-	-
14	5/0/0	5/0/1	10/0/4	-	-	-	-

collected on D439 was toxic, 2 were seronegative and 2 were seropositive, with VN titres of 1/40 and >1/40, respectively. Two of the 19 sera collected on D504 were also seropositive, with VN titres of 1/40 and >1/40, respectively.

Real time RT-PCR testing

RRT-PCR was performed on serum samples collected between D139 and D253, while heart samples were collected and tested from D168 to D253. A total of 32 sera (3.5%) were RRT-PCR-positive. With the exception of a single serum collected on D244, all these were VN-negative. Excluding 1 viraemic serum detected on D176, all viraemic sera were also RRT-PCR-positive. At all sample points, the sensitivity of RRT-PCR on sera was as good or better than screening for viraemia in cell culture (Fig. 1). The peak prevalence of positive sera by RRT-PCR was 35% on D153. Thereafter, values ranged from 25 to 0%, with 2 positive sera detected on D253. RRT-PCR results for heart were available for D199 to D244. Three samples (6%) were positive, comprising 2 samples collected on D199 and 1 on D211. One of these came from a viraemic, seronegative fish, the other 2 from non-viraemic, seropositive fish, with VN titres of >1/40.

Histopathology

Histological examination was initiated when viraemia was detected on D146. The samples collected the previous week (D139) and all subsequent samples were

examined and the results are summarised in Table 2. Overall, the histological lesions observed were mild. The predominant lesion observed was mild chronic focal to multifocal myocardial inflammation with associated infiltration of mononuclear cells (Fig. 2). This was present in both layers of the heart, although more evident in the spongy ventricular muscle. In some cases it was accompanied by focal to diffuse epicarditis associated with a thickened, fat-laden epicardium. These cardiac lesions were observed in between 3 and 5 fish at 13 sample points between D139 and D244, being seen less frequently in samples from the final 2 sample points.

Lesions diagnostic for PD were observed on only 2 occasions. Mild chronic PD lesions were observed in the tissues of 1 of 5 fish on D146, the first occasion that viraemia and VN antibodies were detected. These tissues came from the only seropositive fish sampled on D146. The pancreas of this fish was at the recovery stage, and there was mild myofibrillar degeneration in the heart, RM and WM. At the next sample point (D153), when the peak prevalence of viraemia was observed, 2 of 5 fish had diffuse pancreatic necrosis, typical of acute PD. Both these fish were RRT-PCR-positive, with 1 also being viraemic. Significant pancreatic atrophy was observed in a single fish on D320. Apart from these, the pancreas of all remaining fish examined appeared normal. No RM lesions were observed in any other fish examined. Mild, single cell WM hyaline degeneration was observed in 2 fish, 1 on D211 and 1 on D237, and mild WM lesions were also seen at some other sample points also (Table 2). Mild focal to multifocal gliosis of the brainstem was observed in 1 to 3 fish at several sample points between D139 and D229 (Table 2).

Table 2. *Salmo salar*. Frequency of lesions in pancreas (Pan), heart (Ht), red muscle (RM), white muscle (WM) and brain in each of 5 fish at each sampling point. PD: pancreas disease

Day No.	Pan	Ht	WM	RM	Brain	Comments/stage
139	0	3	0	0	1	Non-specific mild cardiac inflammation
146	1	5	1	1	1	Mild cardiac inflammation plus epicarditis; mild chronic PD
153	2	4 ^a	0	0	0 ^a	Mild cardiac inflammation plus epicarditis; acute PD
158	0	4	0	0	1	Mild cardiac inflammation
168	0	4	0	0	2	–
176	0	5	0	0	3	–
192	0	4	1	0	2	Chronic low grade focal inflammation mainly in spongy heart muscle
199	0	5	1	0	0	Mild multifocal cardiac inflammation in both heart layers with fatty inflamed epicardium
206	0	5	0	0	2	Similar to Day 199
211	0	4	1	0	1	Similar to previous weeks with low grade chronic heart lesions and occasional muscle and/or brain lesion
229	0	5	0	0	2	Similar to Day 211
237	0	5	1	0	0	Similar to Day 211
244	0	4	2	0	0	Similar to Day 211
253	0	0	2	0	0	Mild WM degeneration
320	1	1	0	0	0	Significant pancreatic atrophy and mild focal myocardial inflammation

^aOnly 4 sections examined

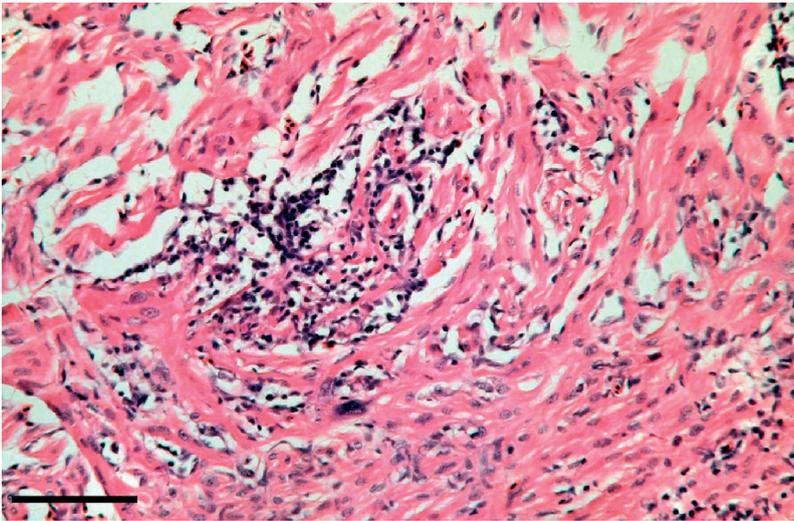


Fig. 2. *Salmo salar*. Chronic mild focal inflammation of ventricular myocardial muscle typical of heart lesions recorded in this study (scale bar = 100 μ m)

To help assess the viral aetiology of the brain and heart lesions, tissues collected at 4 sample points prior to the detection of viraemia (D0, D31, D66 and D104) were examined retrospectively. With the exception of epicarditis in 1 of 5 fish on both D31 and D66, no significant findings were observed.

DISCUSSION

This is the first published prospective longitudinal study of SAV infections in farmed Atlantic salmon, using a combination of virological, serological, RT-PCR and histopathological techniques. This approach allows the progression of infection to be followed at the level of the population rather than of the individual fish.

Despite the high losses attributed to PD in the previous production cycle on this site, infection during this study was subclinical, with no PD-related clinical signs or mortalities noted. Although the occurrence of subclinical infections has been reported before (McLoughlin et al 1998), this is the first time that an outbreak has been described in detail.

In contrast to the findings during clinical outbreaks of PD (McLoughlin et al. 2002, Graham et al. 2005), the histological lesions observed in the current study were mild. As a consequence, pathology sufficient to allow a histological diagnosis of PD was only observed on 2 occasions on D146 (mild chronic PD lesions in 1 of 5 fish) and D153 (acute pancreatic necrosis in 2 of 5 fish). The absence of cardiac and brain lesions in samples collected prior to viraemia (excluding D139) is consistent with their having a viral aetiology. However, they were repeatedly present at higher levels than indi-

cated by serology, virology or RRT-PCR, suggesting that they were not wholly attributable to SAV infection. It cannot be ruled out that the cardiac changes represent normal background pathology, similar to those described by Rodger & Richards (1998).

The absence of changes sufficient to make a histological diagnosis of PD at most sample time points during this study highlights the benefits of screening for VN antibodies and virus by isolation or RRT-PCR as an aid to diagnosis of infection.

The pattern of development of VN antibodies reflected the prolonged period of viraemia and was consistent with slow spread of infection, with seroprevalence only increasing to 15% and beyond from D192, more than 6 wk after infection was first detected. The fact that a maximum of

57.9% of fish in C4 were seropositive, 194 d after infection was first detected, is also consistent with slow spread within this population, although higher seroprevalence figures were noted in some other cages (Table 1). In previous clinical outbreaks in Atlantic salmon, seroprevalence levels of 90 to 100% have been noted within 1 to 3 mo of diagnosis (Graham et al. 2005).

VN antibodies take 2 to 3 wk to develop following infection (McLoughlin et al. 1996). Therefore the finding of a single seropositive fish on D146, when viraemia was first detected indicated that infection had been actually present in this cage for several weeks prior to this. A sample size of 20 gives 95% confidence of detecting 15% prevalence. The failure to detect infection before this (by viraemia, RRT-PCR or serology) suggests that it was present at very low levels and negative results can be attributed to sampling error.

In a previous study (Graham et al. 2006), RRT-PCR was found to be more sensitive than screening for viraemia, and the same observation was made here (Fig. 1). The lower sensitivity of the viraemia screening method is considered to be largely due to the short 3 d virus growth period combined with the small volume of inoculum used. Nevertheless, when used as a screening tool at the population level, screening for viraemia has proved to be a very useful tool for detecting infection. Previous work based on samples from experimental infection has shown that RRT-PCR can detect viral RNA in heart for much longer periods than in serum (D. Graham, unpubl. data). Although the number of heart samples tested was limited ($n = 50$), these findings were supported to some extent in this study, with 6% of heart samples positive compared to 3.5% of sera and 2 of the 3 positive hearts from seropositive fish.

The concept of the basic reproductive rate (R_0) is well established in the epidemiology of terrestrial pathogens (Nokes & Anderson 1988, Anderson & Nokes 1997), defining the average number of secondary cases of infection generated by a primary case in a susceptible population. R_0 is defined as the product of 3 factors: the number of contacts per day made by an infectious individual (κ), the transmission probability per contact (β) and the duration of the infectious period (D). The slow spread of infection in this study, with virus detectable in Cage 4 over an extended period and gradual increase in seroprevalence, suggests that R_0 approximates 1.0, with each fish on average being responsible for the infection of 1 other. This pattern is distinct from that observed in other studies with SAV in both Atlantic salmon and rainbow trout *Oncorhynchus mykiss* (Graham et al. 2006, in press). In particular, in an outbreak of SD in rainbow trout, the period of detectable viraemia lasted only 4 wk, with a peak prevalence of 57.9% during weekly sampling (Graham et al. in press).

The cause of the sub-clinical nature of this outbreak remains unknown. Sequencing studies of a wide range of isolates from salmon and trout have shown that they can be assigned to 1 of 3 subtypes (Weston et al. 2005). To date all UK and Irish salmon isolates have belonged to Subtype 1. Sequencing of isolates from this study is currently being planned to determine if they represent additional, less pathogenic strains. The source of fish was unchanged from the previous production cycle, which tends to exclude host-related factors as the cause of the reduced pathogenicity. Likewise, stocking density is not significantly related to PD-mortality rates (Crockford et al. 1999). Working with another alphavirus, Walton et al. (1973) have shown that epidemic and endemic disease patterns can be related to levels of viraemia. While serum levels of SAV were not determined in this study, it could be hypothesised that in the present study the titre of virus in serum was low, resulting in a consequent low level of viral shedding into the water, ultimately resulting in reduced rates of transmission. Although unusual for alphaviruses, direct transmission of SAV has been shown previously in cohabitant studies (Nelson et al. 1995). Further work is necessary to investigate this.

Examination of data from the other cages (Table 1) indicates that infection was widespread across the site, with infection present in multiple cages soon after initial detection in Cage 4. The notably higher seroprevalence in Cage 13 on D153 suggests that this may have been the index cage on the site, with subsequent onward spread from here. The source of the infection remains unknown. It is possible that a carrier population maintained the infection with spread triggered by a stressor such as grading in November. However, the consistent finding of VN and virus-negative fish over

an extended period from July to early December does not support this. Instead, it points toward an external, unidentified source. One possibility is that infection could have been brought onto the site by the well boat used for the November grading.

The finding of VN activity in saithe comprises the first time that evidence of SAV infection has been found in a non-salmonid species and raises the possibility of saithe being a natural reservoir of infection. This is in contrast to infectious salmon anaemia virus (ISAV), where saithe have been shown to be resistant to infection and not to play a significant role in the epidemiology of the infection (Snow et al. 2002, McClure et al. 2004). Additional work is required to confirm this preliminary finding, particularly in light of the poor specific antibody response typically observed in gadoids to infectious agents (Samuelsen et al. 2006)

Although insect vectors have not been identified for SAV, they form an important part of the epidemiology of other alphavirus infections (Walton et al. 1973, Bowers et al. 1995). Recently, the elephant seal louse *Lepidophthirus macrorhini* has been proposed as the vector for the newly identified alphavirus named southern elephant seal virus (SESV) (La Linn et al. 2001). It can be speculated that the high levels of *Caligus* sp. that appeared on the fish in November represent another, alternative source of infection. High levels of *Caligus* sp. were also reported on fish immediately prior to the outbreak of PD in the previous production cycle on this site. However more work is needed to investigate this.

In conclusion, these results represent the first comprehensive description of a sub-clinical outbreak of PD and highlight the usefulness of RRT-PCR and screening for viraemia and VN antibodies as diagnostic and epidemiological tools. The occurrence of this type of sub-clinical infection emphasises the need to clearly define the status of farms in case-control studies for risk factors for SAV infection. Further studies are required to determine the mechanism(s) responsible for sub-clinical infections and to investigate epidemiology of infections with particular emphasis on the possible role of wild fish.

Acknowledgements. This work was partly funded by EU project QLK2-CT-2001-000970 (Diagnoses, pathogenesis and epidemiologies of salmonid alphavirus diseases). The authors thank Fjord Seafood Scotland site staff for their commitment and help throughout the study.

LITERATURE CITED

- Anderson R, Nokes JD (1997) Mathematical models of transmission and control. In: Detels R, Holland WW, McEwen J, Omenn GS (eds) Oxford textbook of public health, 3rd edn, Vol 2 The methods of public health. Oxford University Press, Oxford, p 689–719

- Bowers DF, Abell BA, Brown DT (1995) Replication and tissue tropism of the alphavirus Sindbis in the mosquito *Aedes albopictus*. *Virology* 212:1–12
- Bruno DW (2004) Changes in prevalence of clinical infectious pancreatic necrosis among farmed Scottish Atlantic salmon, *Salmo salar* L. between 1990 and 2002. *J Fish Dis* 235:13–26
- Castric J, Baudin Laurencin F, Brémont M, Jeffroy J, Le Ven A, Béarzotti M (1997) Isolation of the virus responsible for sleeping disease in experimentally infected rainbow trout (*Oncorhynchus mykiss*). *Bull Eur Assoc Fish Pathol* 17: 27–30
- Crockford T, Menzies FD, McLoughlin MF, Wheatley SB, Goodall EA (1999) Aspects of the epizootiology of pancreas disease in farmed Atlantic salmon *Salmo salar* in Ireland. *Dis Aquat Org* 36:113–119
- Graham DA, Jewhurst VA, Rowley HM, McLoughlin MF, Todd D (2003) A rapid immunoperoxidase-based virus neutralization assay for salmonid alphavirus. *J Fish Dis* 26:407–413
- Graham DA, Jewhurst VA, Rowley HM, McLoughlin MF, Rodger H, Todd D (2005) Longitudinal serological surveys of Atlantic salmon (*Salmo salar* L.) using a rapid immunoperoxidase-based neutralization assay for salmonid alphavirus. *J Fish Dis* 28:373–379
- Graham DA, Taylor C, Rodgers D, Weston J, Khalili M, Ball N, Christie N, Todd D (2006) Development and evaluation of a one-step real-time reverse transcription polymerase chain reaction assay for the detection of salmonid alphaviruses in serum and tissues. *Dis Aquat Org* 70: 47–54
- Graham DA, Jewhurst HL, McLoughlin MF, Branson EJ, McKenzie K, Rowley HM, Todd D (in press) A prospective longitudinal serological, virological and histopathological study of an outbreak of sleeping disease in farmed rainbow trout *Oncorhynchus mykiss*. *Dis Aquat Org*
- Jewhurst VA, Todd D, Rowley HM, Walker IW, Weston JH, McLoughlin MF, Graham DA (2004) Detection and antigenic characterization of salmonid alphavirus isolates from sera obtained from farmed Atlantic salmon, *Salmo salar* L., and farmed rainbow trout, *Oncorhynchus mykiss* (Walbaum). *J Fish Dis* 27:143–149
- Karber G (1931) Beitrag zur kollektiven Behandlung pharmakologischer Reihenversuche. *Arch Exp Pathol Pharmacol* 162:480–483
- Kent ML, Elston RA (1987) Pancreas disease in pen-reared Atlantic salmon in North America. *Bull Eur Assoc Fish Pathol* 7:29–31
- La Linn M, Gardiner J, Warrilow D, Darnell GA and 5 others (2001) Arbovirus of marine mammals: a new alphavirus isolated from the elephant seal louse, *Lepidophthirus macrorhini*. *J Virol* 75:4103–4109
- McClure CA, Hammell KL, Dohoo IR, Gagne N (2004) Lack of evidence of infectious salmon anemia virus in pollock *Pollachius virens* cohabitating with infected farmed Atlantic salmon *Salmo salar*. *Dis Aquat Org* 61:149–152
- McLoughlin MF, Nelson RT, Rowley HM, Cox DI, Grant AN (1996) Experimental pancreas disease in Atlantic salmon *Salmo salar* post-smolts induced by salmon pancreas disease virus (SPDV). *Dis Aquat Org* 26:117–124
- McLoughlin MF, Rowley HM, Doherty CE (1998) A serological survey of salmon pancreas disease virus (SPDV) antibodies in farmed Atlantic salmon, *Salmo salar* L. *J Fish Dis* 21:305–307
- McLoughlin MF, Nelson RT, McCormick JI, Rowley HM, Bryson DB (2002) Clinical and histopathological features of naturally occurring pancreas disease in farmed Atlantic salmon, *Salmo salar* L. *J Fish Dis* 25:33–43
- McLoughlin MF, Peele E, Foyle KL, Rodger HD, O'Ceallachain D, Geoghegan F (2003) An epidemiological investigation of the re-emergence of pancreas disease in Irish farmed Atlantic salmon (*Salmo salar* L.) in 2002. (Mar Environ Health Ser No. 14), Marine Institute, Galway, Ireland
- Munro ALS, Ellis AE, McVicar AH, McLay HA, Needham EA (1984) An exocrine pancreas disease of farmed Atlantic salmon in Scotland. *Helgol Meeresunters* 37:571–586
- Murphy TM, Rodger HD, Drinan EM, Gannon F, Kruse P, Korting W (1992) The sequential pathology of pancreas disease in Atlantic salmon farms in Ireland. *J Fish Dis* 15:401–408
- Nelson RT, McLoughlin MF, Rowley HM, Platten MA, McCormick JI (1995) Isolation of a toga-like virus from farmed Atlantic salmon *Salmo salar* with pancreas disease. *Dis Aquat Org* 22:25–32
- Nokes DJ, Anderson RM (1988) The use of mathematical models in the epidemiological study of infectious diseases and in the design of mass immunization programmes. *Epidemiol Infect* 101:1–20
- Poppe T, Breck O (1989) Pathology of Atlantic salmon *Salmo salar* intraperitoneally immunized with oil adjuvanted vaccine: a case report. *Dis Aquat Org* 29:219–226
- Rodger HD, Richards RH (1998) Observation study of erythrocytic inclusion bodies in farmed Atlantic salmon *Salmo salar* L., in the British Isles. *J Fish Dis* 21:101–111
- Samuelsen OB, Nerland AH, Jørgensen T, Schrøder MB, Svåsand T, Bergh Ø (2006) Viral and bacterial diseases of Atlantic cod *Gadus morhua*, their prophylaxis and treatment: a review. *Dis Aquat Org* 71:239–254
- Snow M, Raynard R, Bruno DW, van Nieuwstadt AP, Olesen NJ, Løvold T, Wallace C (2002) Investigation into the susceptibility of saithe *Pollachius virens* to infectious salmon anaemia virus (ISAV) and their potential role as a vector for viral transmission. *Dis Aquat Org* 50:13–18
- Villoing S, Béarzotti M, Chilmonczyk JC, Brémont M (2000) Rainbow trout sleeping disease virus is an atypical alphavirus. *J Virol* 74:173–183
- Walton EM, Alvarez O Jr, Buckwalter RM, Johnson KM (1973) Experimental infection of horses with enzootic and epizootic strains of Venezuelan equine encephalomyelitis virus. *J Infect Dis* 128:271–281
- Welsh M, Weston J, Borghmans BJ, Mackie D, Rowley H, Nelson R, McLoughlin MF, Todd D (2000) Biochemical characterization of salmon pancreas disease virus. *J Gen Virol* 81:813–820
- Weston JH, Welsh MD, McLoughlin M, Todd D (1999) Salmon pancreas disease virus, an alphavirus infecting farmed Atlantic salmon, *Salmo salar* L. *Virology* 256:188–195
- Weston J, Villoing S, Brémont M, Castric J and 7 others (2002) Comparison of two aquatic alphaviruses, salmon pancreas disease virus and sleeping disease virus, by using genome sequence analysis, monoclonal reactivity, and cross-infection. *J Virol* 76:6155–6163
- Weston JH, Graham DA, Branson E, Rowley HM, Walker IW, Jewhurst VA, Jewhurst HL, Todd D (2005) Nucleotide sequence variation in salmonid alphaviruses from outbreaks of salmon pancreas disease and sleeping disease. *Dis Aquatic Org* 66:105–111