Red vent syndrome in wild Atlantic salmon *Salmo salar* in Scotland is associated with *Anisakis simplex* sensu stricto (Nematoda: Anisakidae)

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ABSTRACT: Simultaneous reports were received between June and July 2007 of wild Atlantic salmon *Salmo salar* with red, swollen, bloody vents returning to geographically diverse rivers in Scotland. By the end of September the condition, colloquially known as 'red vent syndrome' (RVS), was reported from >50 rivers across Scotland. Fish were generally in good overall condition but the vent area showed mild to severe lesions. External characteristics of the syndrome included a swollen, raised, haemorrhagic vent and surrounding tissues, with erosion of the skin, scale loss and moderate to severe bleeding in more advanced cases. Predominantly, the fish affected were 1-sea-winter grilse; however, RVS was also recorded in 2-sea-winter salmon and sea trout *S. trutta*. High numbers of the nematode *Anisakis* Type I larvae were found infesting the discrete region of the vent, a localisation that is reported as novel for the parasite. The hypothesis that this is a different species than that commonly found in the body cavity and viscera was investigated through molecular studies. These studies failed to show evidence that the parasites infesting the vent were different from those in the body cavity, i.e. all were identified as *A. simplex* sensu stricto. No other disease agent was found associated with the lesions or was isolated systemically, and no mortality or prevention of spawning was recorded during the 2007 season. Possible causes, including warming environments in the North Atlantic, are hypothesised as playing a role in the development of RVS in Atlantic salmon.

KEY WORDS: Atlantic salmon · Wild fish · Red vent syndrome · *Anisakis simplex*

INTRODUCTION

Atlantic salmon fishing is an important economic resource, with income generated largely through angling and associated tourism-generated jobs. The last official estimation of the annual income from salmon and sea-trout anglers in Scotland was £73.5 million, supporting an estimated 2200 full-time jobs, mostly in remote, rural areas (Scottish Executive 2004). Public and official concern about wild salmon health status was reflected as early as in the 1930s through studies commissioned by the UK government when the cause and impact of furunculosis was investigated (Mackie et al. 1933); this was followed by studies on 'Dee disease', known later as bacterial kidney disease (BKD), and by the mid 1960s, the condition termed ulcerative dermal necrosis (UDN) was also under investigation (Roberts 1993). It is often very difficult to quantify the effect of a pathogen on wild host populations, and historically, only a few conditions have been reported as causing conspicuous and significant disease epidemics in wild salmon stocks (Bakke & Harris 1998). An example among parasites and probably the most significant in terms

of impact is *Gyrodactylus salaris* (Johnsen & Jensen 1991) affecting Norwegian wild salmon.

Records kept by Marine Scotland (formerly Fisheries Research Services) on wild fish surveys and statutory inspections show that since 2005 there have been sporadic reports of 'red vents' or 'bloody vents' in salmon returning to Scottish rivers. There are also anecdotal observations from anglers and gillies who have fished particular rivers for >20 yr stating that the condition has been consistently observed in the past but more frequently in recent years (D. Stewart pers. comm.) and that it peaked dramatically in 2007. Preliminary observations on red vent syndrome (RVS) in Scotland suggested that the condition was attributable to the presence of the nematode *Anisakis* spp. (Noguera et al. 2008), and Beck et al. (2008) reported similar observations in England and Wales, where the condition showed an increasing trend from 3% in 2005, to 9% in 2006 and up to 27% in 2007 in trap-caught fish screened from River Dee in North Wales. Furthermore, RVS has been observed in salmon from Ireland, Norway and Canada (T. A. Mo pers. comm., H. Rodger pers. comm., G. Seguin pers. comm.). Recently, Iceland has also officially reported the condition (Helgason et al. 2008), and therefore RVS appears to be a feature of the entire North Atlantic region.

The genus *Anisakis* has a pan-global distribution and occurs in all marine environments (Davey 1971, Smith & Wootten 1984). The identification of species within groups was historically done through morphology, but in the late 1980s it was recognised that morphology alone cannot accurately make identifications to the species level; currently, taxonomic issues within the genus *Anisakis* are being further resolved through the development and application of genetic studies. This allowed the demonstration of 2 main clades of species sharing the larval morphology of *Anisakis* spp. Type I or Type II (sensu Berland 1961) (Orecchia et al. 1986, Mattiucci et al. 2005, Mattiucci & Nascetti 2006, Valentini et al. 2006). Moreover, genetic markers from multilocus allozyme electrophoresis have shown that *A. simplex* (Davey 1971) falling in the first clade is a complex of sibling species comprising *A. simplex* sensu stricto (s.s.), *A. pegreffii* and *A. simplex* C (Nascetti et al. 1986, Mattiucci et al. 1997, D'Amelio et al. 2000, Mattiucci & Nascetti 2006), They not only differ in their genetic structure, but also in their life-history and geographic distribution, with *A. simplex* s.s. being reported to be widespread between 35° N and the Arctic polar circle of both the western and eastern waters of both the Atlantic and the Pacific oceans, with a southern limit of the eastern North Atlantic in the waters of the Gibraltar area (Mattiucci et al. 1997, D'Amelio et al. 2000, Abollo et al. 2001, Mattiucci & Nascetti 2006). The current taxonomy and ecology of the recognised species have been recently reviewed by Mattiucci & Nascetti (2008).

The parasite has a complex life cycle involving marine mammals (especially cetaceans) as the final host, and fish, squid and planktonic crustaceans as paratenic or intermediate hosts harbouring the larval stages (Smith & Wootten 1978, Mattiucci et al. 2004). Humans can also accidentally become infected by eating raw or undercooked seafood or cephalopods that contain the third-stage larvae (L3), which can be invasive, penetrating the host's stomach or intestinal wall and causing gastroenteric manifestations or inducing allergic reactions (Zaffiro et al. 2002). Among the roundworms that infect fish, members of the genus *Anisakis* are by far the most widespread and abundant parasite and are found in almost all commercially exploited species in North Atlantic waters (Smith & Wootten 1978, Wootten & Cann 1980). The larval worms (L3) penetrate the gut wall and normally encapsulate in the body cavity tissues and the external surfaces of the gut, pyloric caeca, liver and fat tissue, inducing a mild to moderate adhesion reaction in the host. However, they can also migrate and locate in the skeletal muscle thereby affecting the edible part of the fish (Smith & Wootten 1984, Abollo et al. 2001). As a chronic infection *Anisakis* spp. does not result in mass mortality (Kent & Fournie 1993). However, it is one of the most important parasitic problems for the fishing industry because it reduces the quality of the flesh and can have an impact on human health (zoonotic condition). *Anisakis* spp. are at the top of the list of pathogenic roundworms transmitted via seafood products (Audicana et al. 2002, Huss & Embarek 2003).

The widespread nature and almost synchronised reporting of RVS prompted the present study, with the aims of investigating the aetiology of RVS and responding to questions on the possible impact on wild fish spawning and whether farmed fish were affected by the condition. Warming environments induced by climate variability in the North Atlantic is hypothesised as playing a role in the occurence of RVS.

MATERIALS AND METHODS

Geographical distribution. Reports received at the Marine Laboratory at Marine Scotland between late June and September 2007 were used to map the rivers or catchments areas reporting RVS-positive fish. The map was produced using the geographic information systems (GIS) software ESRI.

Sample collection. Between July and September 2007, 31 fish were collected for sampling purposes. Fifteen were caught using bag nets at Strathy Point (Sutherland), including 3 fish exhibiting normal vents

for use as reference material. A further 12 fish were collected using a sweep net at the North Esk (Angus) upper estuarine fishery, and the remainder were provided by anglers, 2 from River Dee (Aberdeenshire), 1 from River Don (Aberdeenshire) and 1 from River Inver (Sutherland). Fish necropsy was generally completed on site, with collection of specific samples for haematological, virological, bacteriological, histopathological, parasitological and molecular analysis. Sampling date, origin, number of fish and sex are shown in Table 1.

Spawning success was further analysed in an additional 11 broodstock fish held at 3 different locations during the 2007 stripping season: the hatchery at River Don Mill of Newe, the Conon fish trap (managed by the District Salmon Fishery Board) and the River Dee trap at Baddoch (managed by the Marine Scotland, Freshwater Laboratory) (Table 1). Marine Scotland Fish Health Inspectorate (FHI) records of statutory testing on salmon sites were also analysed for any reported case of RVS- or *Anisakis* spp.-affected fish.

External examination. All fish were macroscopically examined and photographed prior to necropsy, and tissue was sampled at the point of capture, with the exception of those provided by anglers, which were received and sampled in the laboratory.

Photographs of the vent area were used to build a graphical key of the condition based on the lesions described and the tissues involved, and the RVS-affected fish were categorised as mild, moderate or severe (see Fig. 3).

For the purpose of this description, under the term 'vent' (specifically the external opening of the alimentary canal, the anus), we will refer to all tissues effectively involved in RVS: the vent and urogenital papilla, the genital cavity and pore, the last portion of the urinary canal, as well as the surrounding tissues of the

Table 1. Sampling date, origin, numbers and sex of analysed *Salmo salar* (**bold**: analysed for spawning success). R: river; nd: not determined; M: male; F: female; Gr: grilse

Date (dd/md/yy)	Region	n	Sex
03/07/07	Strathy Point	1	nd
25/07/07	Strathy Point	1	M
28/07/07	R Dee/Banchory	1	nd
02/08/07	Strathy Point	13	4 M, 1 F, 8 nd
13/08/07	R North Esk	6	2 F, 4 M
14/08/07	R North Esk	6	1 F Gr, 1 F, 4 M
04/09/07	R Inver	1	nd
15/09/07	R Dee/Maryculter	1	nd
29/09/07	R Don	1	M
06/11/07	R Dee/Baddoch	**1**	**F**
22/11/07	R Mill of Newe	**5**	**F**
27/11/07	R Conon/Hatchery trap	**5**	**3 F, 2 M**

posterior abdominal wall, abdominal pores and the skin and underlying adipose and muscle layers in the immediate area.

Blood smears. Caudal-vein blood samples were collected from Strathy Point and North Esk fish using Vacuette tubes (Greiner Bio-One). Blood smears were prepared on site, air-dried and transported to the laboratory, where they were Giemsa-stained and screened for evidence of blood-born parasites, bacterial infection and the presence of erythrocytic inclusion bodies.

Bacteriology and virology. For bacteriological assessment, an inoculum from kidney, vent lesion and hindgut was streaked onto blood agar (BA; Oxoid), trypticase soy agar (TSA; Oxoid), TSA plus 1.5% NaCl (Oxoid), Müller-Hinton agar (MHCA; Difco) with 0.1% L-cysteine added during preparation and Anacker & Ordal agar (A&O) prepared in the laboratory using 0.5% tryptone (as modified by Lorensen 1993). Plates were incubated at 15°C (A&O) or 22°C (TSA, TSA + NaCl, MHCA) and checked for growth daily for 7 d or up to 12 wk for MHCA. Imprints from the kidney and the vent lesion were also taken, air-dried and transported to the laboratory, where they were Gram-stained and examined microscopically for bacterial and rickettsial agents.

For virological assessment, tissue samples were aseptically dissected, and pooled material from the head kidney, spleen, heart and encephalon from each fish were collected into separate viral transport medium tubes with Leibovitz-15 buffered medium with 10% newborn calf serum plus antibiotics (gentamicin, polymyxin B and penicillin/streptomycin); pH was adjusted to 7.4 through the addition of 1 M NaOH. Samples were homogenised and clarified by sedimentation and inoculated into TO, BF-2, FHM, CHSE-214 and E-11 cell lines. Cultures were incubated at 15°C for 7 to 14 d and were checked for cytopathic effects (CPE) at the end of the incubation period. All cultures were subcultivated to fresh cells with a further 7 to 14 d incubation period depending on cell line and read microscopically for CPE at the end of this period (OIE 2006).

Molecular viral detection. RNA was extracted from kidney and vent tissue sampled in RNAlater (Ambion) using the MagAttract RNA Tissue Mini M48 kit and the BioRobot M48 extraction workstation (Qiagen). Tissues were screened for the presence of alphavirus using a previously described method (Hodneland & Endresen 2006). Briefly, cDNA was generated using TaqMan Reverse Transcription Reagents according to the manufacturer's instructions (Applied Biosystems). Real-time PCR was performed in a 20 μl final volume containing 1 μl cDNA, 1× SensiMix Buffer (Quantace), 2.5 mM MgCl$_2$, 0.02 units uracil-N-glycosylase (UNG), 0.9 μM of each primer and 0.25 μM probe on an ABI 7000 real-time PCR machine.

Molecular parasite identification. Nematodes removed from the body cavity and vent tissue were stored in 100% ethanol. The parasite's outer coating was removed to reduce host-tissue contamination prior to DNA extraction using the DNeasy Tissue Kit (Qiagen) or by direct tissue lysis (Cunningham et al. 2001). Internal transcribed spacer ribosomal DNA (ITS-rDNA) was amplified as described (Zhu et al. 1998), and the resultant PCR products were purified by MinElute Gel Purification (Qiagen) and sequenced on a CEQ 8800 using the GenomeLab DTCS Quick Start kit (Beckman Coulter). Restriction fragment length polymorphism (RFLP) analysis was performed on purified ITS PCR products using *Hinf*I and *Hha*I (D'Amelio et al. 2000, Abollo et al. 2003, Pontes et al. 2005, Umehara et al. 2007). PCR products were cloned using the TOPO TA cloning vector (Invitrogen). Partial cytochrome oxidase I (*cox1*) mitochondrial DNA (mtDNA) was also amplified and sequenced from a subset of nematodes as described in Cross et al. (2007).

Anisakid ITS rDNA and *cox1* sequences were aligned using Bioedit vers. 7.0.5.3 (Hall 1999). Phylogenetic relationships of *cox1* sequences were determined using PAUP (vers. 4.0B10; Swofford 1999) and maximum-likelihood (ML) analysis with likelihood parameters determined by ModelTest (Posada & Crandall 1998). Support for relationships was tested using neighbor-joining (NJ) analysis (ML settings) and 1000 replicates.

Nematodes from 2 clinically affected salmon were analysed with the purpose of comparison between body cavity and vent larvae. Four and 20 parasites from the vent area and 2 and 20 from the body cavity from each fish respectively were analysed by ITS-RFLP. ITS and *cox1* regions were sequenced from 14 and 6 vent nematodes and 12 and 6 body cavity nematodes respectively.

Histology. Samples from all 42 fish were taken for histopathological assessment by light microscopy. Tissues were fixed in 10% neutral buffered formalin for 24 to 48 h, and later trimmed, routinely processed and paraffin-embedded. Sections of 3 to 4 μm were routinely stained with haematoxylin and eosin (H&E), periodic acid-Schiffs (PAS) and Giemsa. Parasites in tissue sections were identified as nematodes by observing the multilayered acellular cuticle, lateral chords and pseudocoelom, according to Bruno et al. (2006).

Parasitology. For parasitological analysis, pieces of foregut, hindgut and vent tissue were fixed in 100% ethanol and any parasites were later dissected from the tissue. Individual nematodes were also removed from body cavity viscera and in all cases they were analysed and identified morphologically after Berland (1961) and using keys from Moravec (2004).

RESULTS

The earliest Marine Scotland records describing a 'red swollen vent' on wild Atlantic salmon were found in 3 routine diagnostic cases from 2005, followed by one in 2006 during a statutory visit, though no specific samples were taken from these cases. During 2007, the simultaneous observations from all across Scotland resulted in the analysis of RSV distribution and targeted sample collection.

Geographical distribution

Between late June and the end of September 2007 a total of 59 rivers or catchment areas across Scotland were reported positive for RVS. The geographical distribution is presented in Fig. 1. All fish collected at Strathy Point were from the sea, and those from the North Esk had just returned from the sea into freshwater. Both male and female Atlantic salmon were represented among the analysed fish. It should be stressed that this only represents sample sex distribution, as the fish analysed for the purpose of the present study will not be suitable for any extrapolation, due to potential biases in sampling, with some cases originating from angling and the majority being a subsample of a larger, non-assessed stock (Table 1). The laboratories' records show that no farmed salmon sites registered any case of fish affected by the syndrome or any similar condition affecting the vent area, so no evidence was found of RVS affecting farmed salmon.

Gross observations

The fish displaying signs of RVS were primarily 1-sea-winter grilse weighing between 2 and 4 kg, although 2 reports of 2-sea-winter Atlantic salmon *Salmo salar* and a single sea trout *S. trutta* were also recorded. The only external lesions observed were erythema due to the grazing action of sea lice around the anal fin and the swollen, haemorrhagic area around the vent and urogenital papilla (Fig. 2a). Mildly affected fish showed a generalised reddening around the vent and urogenital openings, sometimes with discrete petechial haemorrhage but limited swelling of the area (Fig. 3a); the moderate stage comprised a more widespread erythema in the surrounding 25 to 30 mm area with associated mild swelling, with conspicuous foci of petechial haemorrhage and scale lifting (Fig. 3b). The severe stage of the condition comprised a marked protrusion of the vent area, with generalised haemorrhage and swelling, and breakdown of the skin surface, scale loss and bleeding (Fig. 3c).
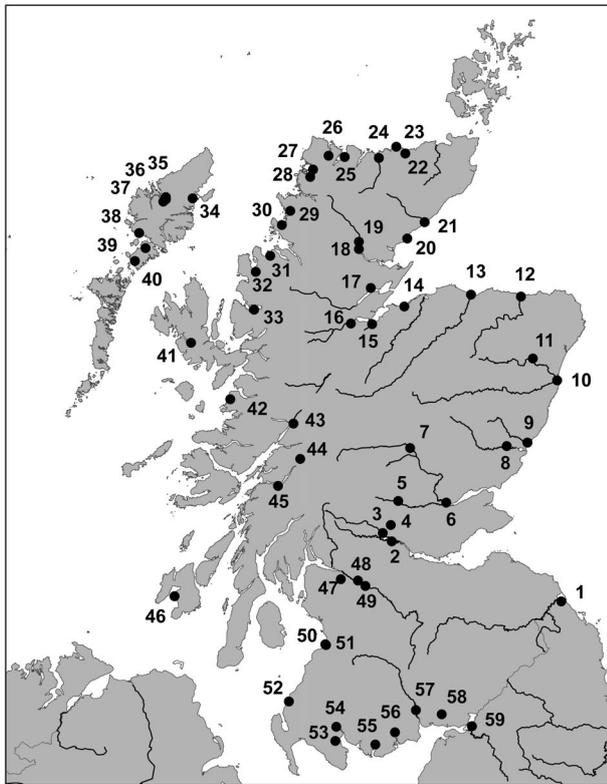
Fig. 1. Distribution of reported red vent syndrome (RVS)-affected rivers or catchments areas in Scotland during the period June to November 2007. 1, Tweed; 2, Forth; 3, Teith; 4, Allan; 5, Earn; 6, Tay; 7, Loch Faskally; 8, Esk (South); 9, Esk (North); 10, Dee (Aberdeenshire); 11, Don; 12 Deveron; 13, Spey; 14, Nairn; 15, Ness; 16, Beauly; 17, Averon; 18, Oykel; 19, Shin; 20, Brora; 21, Helmsdale; 22, Hallidale Burn; 23, Strathy Point (netting station); 24, Naver; 25, Hope; 26, Dionard; 27, Rhiconich; 28, Laxford; 29, Inver; 30, Garvie; 31, Gruniard; 32, Ewe; 33, Balgy; 34, Abhainn Ghrioda; 35, Gearraidh na h-Aibhne; 36, Abhainn Dhubh; 37, Abhainn Ghriomarstaidh; 38, Abhainn Eabhal; 39, Abhainn Lacasdail; 40, Loch Moracha; 41, Drynoch; 42, Morar; 43, Lochy; 44, Etive; 45, Awe; 46, Lagan; 47, Gryfe; 48, Kelvin; 49, Clyde; 50, Ayr; 51, Doon; 52, Stinchar; 53, Bladnoch; 54, Cree; 55, Dee (Kirkcudbrightshire); 56, Urr; 57, Nith; 58, Annan; 59, Esk (Border)

All fish were found to be in generally good external condition, and a moderate to high fat storage in the body cavity could be observed at necropsy in all fish. Some fish showed signs of recent feeding, though most did not, as confirmed through empty guts and enlarged gall bladders. Fish analysed had low to moderate numbers of nematode larvae within the body cavity, around the pyloric caeca-pancreas and on the liver surface, but parasites were not obvious from the stomach to the end of body cavity (Fig. 2b). Those fish with swollen vents also had numerous larvae aggregated within the discrete region of the vent-urogenital papilla, and on some occasions, parasites could be seen with the

naked eye at the surface of the vent through the transparent scales (Fig. 4a). When the skin was carefully dissected and lifted, several larvae were observed right underneath the tegument between the hindgut and the skin (Fig. 4b). Parasite burden could be very large on symptomatic fish, with a count of >100 larvae within the vent region from a single salmon dissected ad hoc.

Histology

All fish with mild, moderate or severe lesions as well as 2 of the reference normal fish harboured nematode larvae in the vent region. Summer estuarine fish from Strathy Point and the North Esk showed capillary dilation, blood congestion, haemorrhage and moderate to severe inflammation (Fig. 5), dominated by eosinophilic granular cells (EGCs) (Fig. 6), melanomacrophages and occasionally, multinucleated giant cells (Fig. 7), mostly associated with non-encapsulated parasites. EGCs decreased to very low numbers or were absent in fish sampled several months later in freshwater (spawned autumn fish). These fish showed predominantly encapsulated larvae within a granulomatous connective tissue reaction (Fig. 8) and increase in melanin-containing cells (both melanomacrophages and melanophore-like cells) (Fig. 9). Degeneration of encapsulated larvae associated with necrosis and inflammatory reaction was occasionally observed (Fig. 10). The epidermis of severely affected summer fish could be absent or largely detached from the basal membrane, while autumn fish showed a completely healed epidermal layer. The dermal layer of both summer and autumn fish harboured a number of larvae, and was seen infiltrated and haemorrhagic in the early summer fish, while in the autumn fish, this region was thickened due to reparative processes with some inflammatory cells and regenerative scales (Fig. 11). Parasite larvae were localised immediately outside the terminal portion of the hindgut (rectum), ventro-laterally in the discrete space towards the skin, between the hindgut and the genital canal/cavity (depending on the section level), between the genital cavity and the urethra, and dorso-laterally to the urogenital ducts deep within the skeletal musculature (Fig. 11). Inflammatory reaction, when seen in this latter location, was apparently less severe. Non-encapsulated larvae were occasionally seen within the lumen of the genital cavity of the recently spawned fish (Fig. 12). Capillary congestion and surrounding increased free blood was observed, likely as a result of the assisted stripping process.

Most fish examined also had larvae on the liver surface and encysted around the spleen and the stomach wall with an associated moderate adhesion reaction.
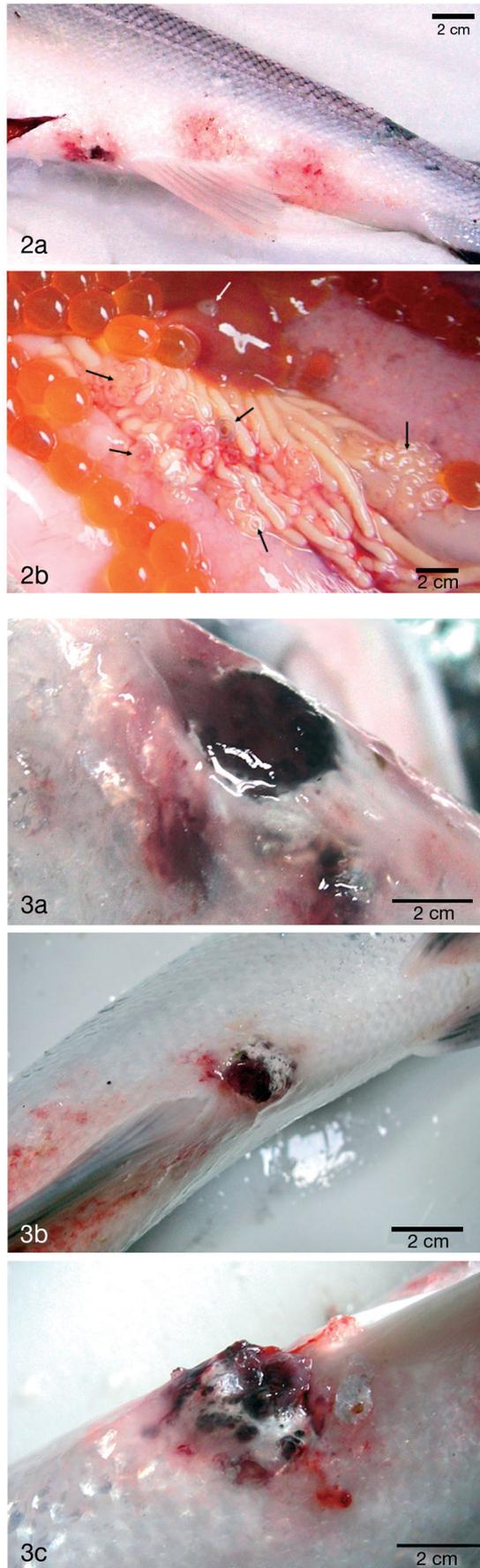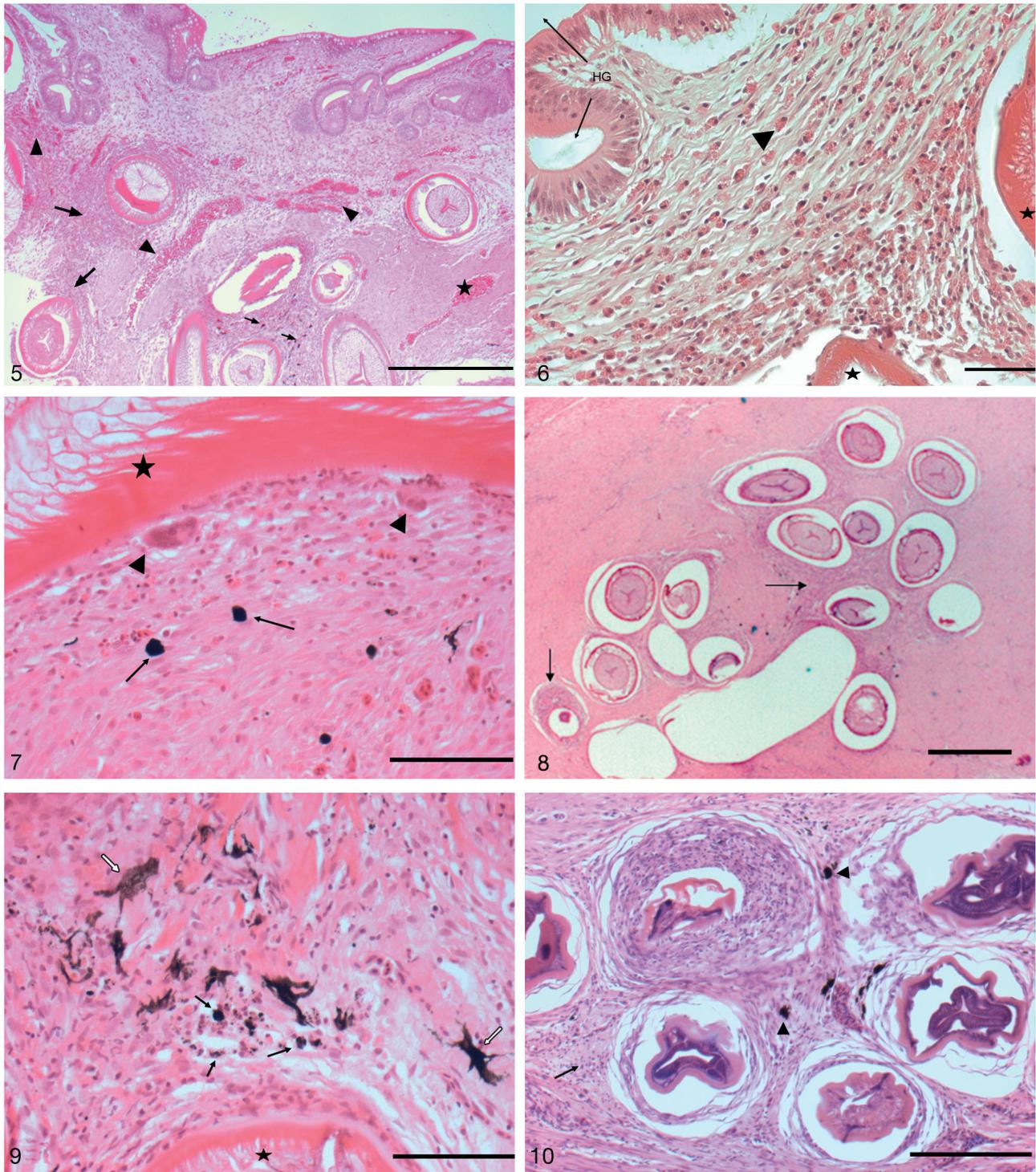
Figs. 2 to 4. *Salmo salar* affected by red vent syndrome (RVS). Fig. 2. (a) Typical external observations of fish from estuarine waters and (b) internal aspect of a freshwater autumn fish. Note larvae (arrows) on liver, stomach and around the pyloric caeca. Fig. 3. Graphical key of RVS condition based on lesions described and tissues involved: (a) mild, (b) moderate and (c) severe. Fig. 4. Parasite larvae could occasionally be seen with the naked eye even in fish not severely affected. (a) Larvae (arrows) just at the surface of the vent. (b) With tegument lifted, numerous larvae (★) seen between hindgut (arrow) and directly underneath the skin

EGCs were increased in the lamina propria and also widespread in the gills and bulbus arteriosus of summer fish (Fig. 13). Gill EGCs were seen surrounding the lamellar rod, de-granulating and inducing an apparent re-absorption of the cartilage. EGCs dropped to almost zero in tissues from the autumn fish. Other observations included one fish with a cestode within the gut lumen and another one with protozoans in the kidney tubules. All other tissues were within the normal range, i.e. none of the fish showed a systemic compromise of any of the organs normally targeted in infectious disorders, including the kidney, liver, spleen, gut mucosa, pancreas and heart.

Microbiology, haematology and parasitology

Isolates from the hindgut reflected abundant normal gut flora with no differences between those from fish with or without vent lesions. No bacteria were ob-

Figs. 5 to 10. *Salmo salar* affected by red vent syndrome. <u>Fig. 5.</u> Capillary dilation, blood congestion (★), haemorrhage (arrowheads) and inflammation (large arrows). Melanomacrophages are also present (small arrows). H&E. Scale bar = 500 µm. <u>Fig. 6.</u> Inflammation massively dominated by an influx of eosinophilic granular cells (EGCs) (arrowhead) in the summer fish. Parasite larvae (★), hindgut (HG). H&E. Scale bar = 100 µm. <u>Fig. 7.</u> Melanomacrophages (arrows) and multinucleated giant cells (arrowheads) associated with non-encapsulated parasites (★). H&E. Scale bar = 100 µm. <u>Fig. 8.</u> Granulomatous tissue reaction encapsulating the larvae (arrows). Scale bar = 500 µm. <u>Fig. 9.</u> Melanin-containing cells: melanomacrophages (arrows) and melanophore-like cells (white arrows). Parasite larvae (★). H&E. Scale bar = 100 µm. <u>Fig. 10.</u> Necrosis and inflammatory reaction (arrow) associated with degenerative parasite larvae (see melanomacrophages; arrowheads). H&E. Scale bar = 200 µm
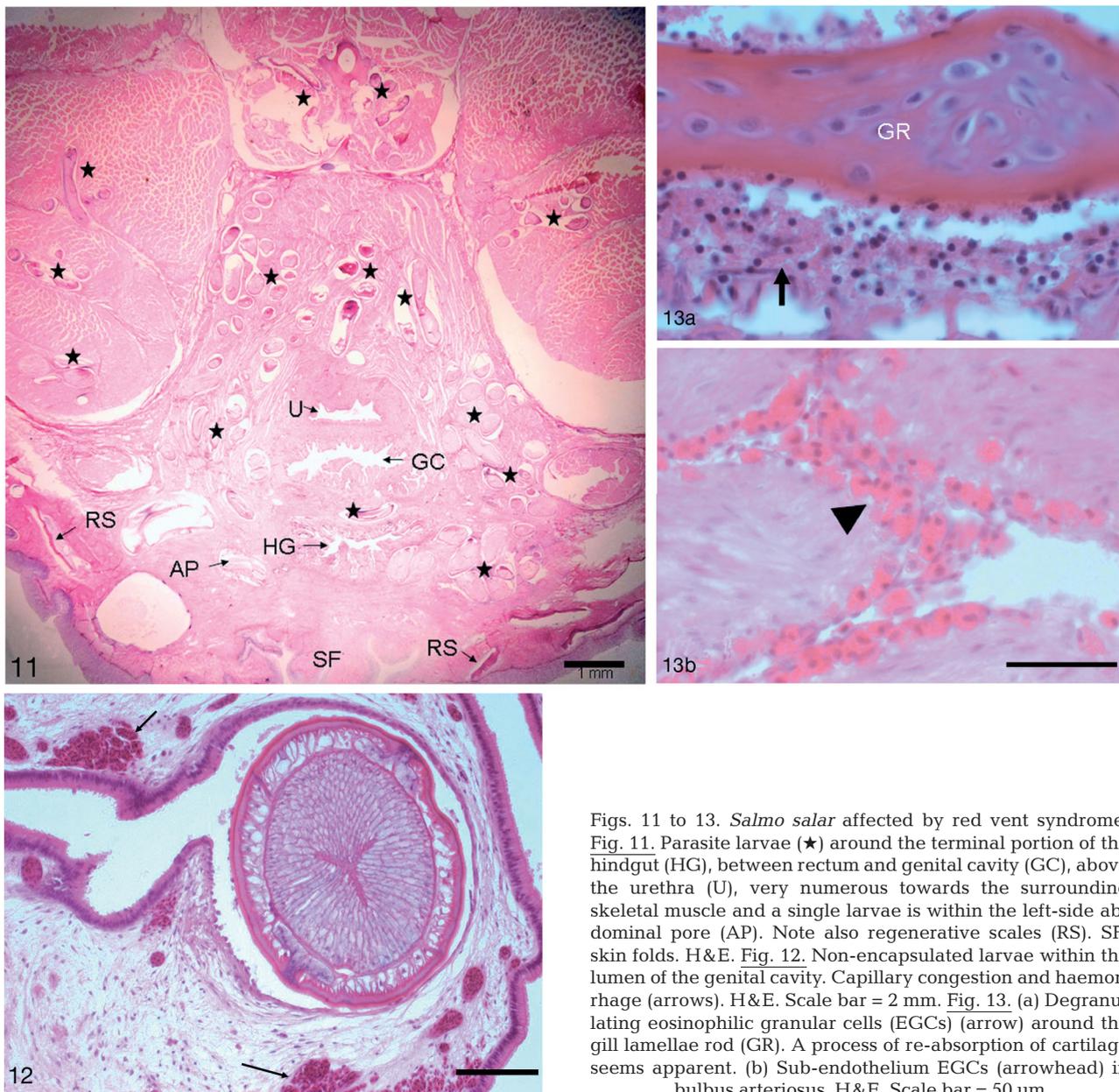
Figs. 11 to 13. *Salmo salar* affected by red vent syndrome. Fig. 11. Parasite larvae (★) around the terminal portion of the hindgut (HG), between rectum and genital cavity (GC), above the urethra (U), very numerous towards the surrounding skeletal muscle and a single larvae is within the left-side abdominal pore (AP). Note also regenerative scales (RS). SF: skin folds. H&E. Fig. 12. Non-encapsulated larvae within the lumen of the genital cavity. Capillary congestion and haemorrhage (arrows). H&E. Scale bar = 2 mm. Fig. 13. (a) Degranulating eosinophilic granular cells (EGCs) (arrow) around the gill lamellae rod (GR). A process of re-absorption of cartilage seems apparent. (b) Sub-endothelium EGCs (arrowhead) in bulbus arteriosus. H&E. Scale bar = 50 µm

served in either Gram-stained vent-tissue smears or Giemsa-stained slides, which correlated with the negative culture results.

Virological testing as well as bacterial cultures from kidney and vent tissue were also negative for all notifiable disease agents.

The examination of blood smears was negative for blood-borne parasites and erythrocytic inclusion bodies.

The majority of nematode larvae collected were alive and displaying motility, and measured between 10.2 and 20.2 mm in length. Larvae were identified morphologically as *Anisakis* Type I larvae (sensu Berland 1961). Some fish had a few parasites identified as *Hysterothylacium gadi aduncum* (Moravec 2004) in the posterior hindgut.

Molecular analysis

Molecular analysis identified the *Anisakis* spp. larvae from both cavity and vent as *A. simplex* s.s., based on *Hinf*I and *Hha*I RFLP patterns (Fig. 14) (D'Amelio et al. 2000) and sequence data. RFLP patterns were identical for all parasites from both vent and cavity. A faint band at approximately 700 bp in the *Hinf*I digest was observed in all samples from vent and cavity, and
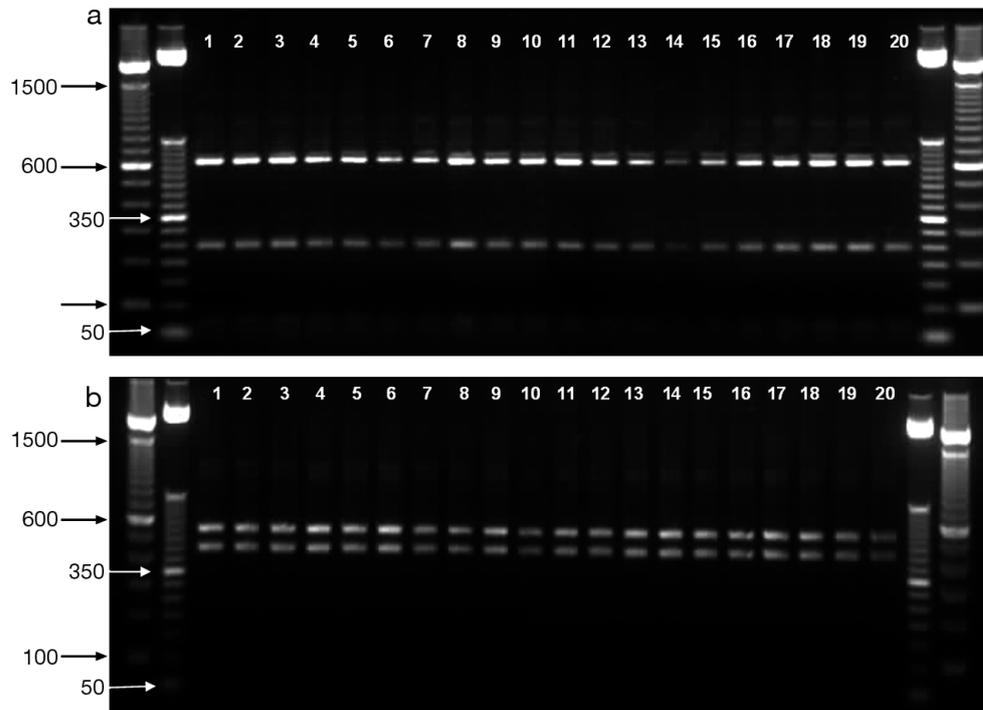
Fig. 14. (a) *Hinf*I and (b) *Hha*I internal transcribed spacer-restriction fragment length polymorphism (ITS-RFLP) patterns of *Anisakis* spp. parasites collected from vent tissue (Lanes 1 to 10) and cavity (Lanes 11 to 20) of Atlantic salmon displaying red vent syndrome

may represent incomplete digestion. All *Anisakis* spp. specimens sequenced had nucleotides (GenBank accession no. GQ143710) identical to that of *A. simplex* s.s. (AY826723; D'Amelio et al. 2000) at those positions which vary between the ITS of known species (AB196671, AB277822, AJ937669, AY821738, AY821739, AY821740, AY821749), apart from 3 specimens which displayed a C/T heterogeneity at position 305 (GQ143709) and a single specimen which displayed a C/T heterogeneity at position 305 and also at position 173 (GQ143711). *A. simplex* s.s. AY826723 has a T at position 305, while some other species, including *A. simplex* C and *A. pegreffii* have a C. These 4 specimens displaying heterogeneity originated from both vent (n = 3) and cavity (n = 1); all specimens displayed heterogeneity at position 791 (C/T). All nucleotide positions are given relative to the sequence for AY826723. The heterogeneity observed in sequenced PCR products was confirmed for positions 173 and 305 by sequencing of cloned products, but not for position 791, where all 4 clones analysed contained a C. The observed heterogeneity at position 791 may represent a sequencing artefact. Cloned ITS sequence contained C and T, T and T and C and C at positions 173 and 305 respectively. A T and C variant was not observed. Based on alignment of available sequences from Gen-Bank, the heterogeneity observed in the present study

did not represent potential hybrids of known anisakid species with respect to displaying polymorphism at all species-diagnostic sites. However, sequences from 2 *A. simplex* specimens infecting herring *Clupea harengus membras* in the Baltic Sea (AJ937670/71) displayed T at position 173, and a specimen from Baltic Sea cod displayed C/T (AJ225065) at this position. These sequences did not show polymorphisms corresponding to position 305 as seen in some *Anisakis* spp. specimens analysed from RVS-affected salmon. Recently an ITS rDNA sequence from *A. simplex* infecting onion-eye grenadier *Macrourus berglax* from the east coast of Greenland was submitted to GenBank (GQ131690). This sequence had N at position 173 and Y at position 305. An overview of ITS rDNA sequence variation in *A. simplex* is given in Fig. 15.

Twelve different *cox1* haplotypes were obtained, differing from each other by 3 to 15 nucleotides over a region 955 nucleotides in length (GQ132122 to GQ132133). No consistent differences or groups associated with vent or cavity sampling site, or with the presence of polymorphisms in the ITS rDNA were observed, neither by visual examination of the aligned sequences nor following phylogenetic analysis. Most variation consisted of nucleotide polymorphisms confined to individuals. Two positions showed a consistent difference between the anisakids analysed from

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                p173  p305                                    p791
                  ↓ ↓                                          ↓
RVS    GQ143710  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--NAA CGCAACGAAC GG--TATT
RVS    GQ143709  TC--TGYA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
RVS    GQ143711  TC--YGYA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sim  AY826723  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--NATT
A.sim  AJ937671  TC--TGTA TA-CAGTTC- -GTGTGATC- TATGT--CGA CGCAACGAAC GGG-TATT
A.sim  AJ937670  TC--TGTA TA-CAGTTC- -GTGTGATC- TATGT--CGA CGCAACGAAC GGG-TATT
A.sim  AJ225065  TC--YGTA TA------- ---------- ---------- ---------- --------
A.sim  GQ131690  TC--NGYA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sim  AJ937669  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--CGA CGCAACGAAC GGG-TATT
A.sim  EU718471  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sim  GQ131692  TCT-CGTA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACNNAC GG--TANA
A.sim  GQ131691  TC--CGTA TW-CAGTTC- -GTGTGATC- TANN---NAM NCNN--NAAC GG--T-TT
A.sim  AB196672  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sim  EU624342  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sim  AF411202  TC--CGTA TA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sim  AJ225066  -------- -------TC- -GTGTGATC- TATGT--CAA CGCAACGAAC GGG-TATT

A.peg  AY826720  TC--CGCA CA-TNATTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.peg  AY603531  TC-GYGCA CAGCAGTKCC GCYAYSAYC- TA-CTSTCAA CGYTACGACA CTCGTATT
A.peg  AM706346  CC--CACG CA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GGG-TATT
A.peg  EU873166  -------- ------CTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT

A.simC GQ167200  TT--CGCA TA-CAGTCC- -GTGTGCTT- TGTGT--CAA CGCAACGAAC GG--TATT
A.simC GQ131689  TC--CGCA TA-CAGTCC- -GTGTGCTT- TATGT--CAA CGCAACGAAC GG--TATT
A.simC AY821736  TC--CGCA TA-CAGTCC- -GTGTGCTT- TRTGT--CAA CGCAACGAAC GG--TATT
A.simC AY826722  TC--CGCA TA-CAGTCC- -GTGTGCTT- TATGT--CAA CGCAACGAAC GG--TATT

A.sp.  AY821749  TC--CGTA TA-CAGTTT- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sp.  AY821745  TC--CGCA CA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
A.sp.  AB246367  TC--CGCA CA-CAGTTC- -GTGTGATC- TATGT--CAA CGCAACGAAC GG--TATT
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Fig. 15. Alignment of internal transcribed spacer ribosomal DNA (ITS rDNA) sequences from *Anisakis* spp. Nucleotide positions conserved across all sequences have been removed. Nucleotides differing between sequences reported as *A. simplex* (*A. sim*) are in **bold**. Positions (p) 173 and 305, which displayed heterogeneity in *Anisakis* spp. specimens from salmon with red vent syndrome (RVS), are indicated. Nucleotide positions that differ consistently between different *Anisakis* spp. and *A. simplex* s.s. are highlighted in grey. *A. peg*: *A. pegreffii*, *A. sim*C: *A. simplex* 'C', *A.* sp.: uncharacterised *Anisakis* species

salmon with RVS in the present study, and *cox1* from *Anisakis simplex* s.s. infecting herring populations around the British Isles (Cross et al. 2007). These nucleotide differences translated to differences at the amino acid level. A number of differences were observed with *cox1* from *A. pegreffii* (Fig. 16).

DISCUSSION

Although the greatest diversity of parasites in Atlantic salmon *Salmo salar* are known from their marine phase, few are likely to be of pathogenic significance. Conclusive evidence is lacking though, as parasitised salmon cannot be readily identified and tracked (Bakke & Harris 1998). The present investigation into abnormal, haemorrhagic vents in returning wild Atlantic salmon showed that the discrete region of the vent and urogenital papilla is affected by a parasite-induced condition associated with the presence of high numbers of *Anisakis simplex* s.s. larvae. No bacterial, viral or fungal pathogen was found to be associated with the condition. The distribution, covering virtually all Scottish salmon rivers and catchment areas

Fig. 16. Alignment of partial anisakid cytochrome oxidase I (cox1) mitochondrial putative protein sequence. RVS: anisakids isolated from salmon displaying red vent syndrome (present study). C: cavity, V: vent. *cox1 sequence representative of individuals showing polymorphism in their internal transcribed spacer ribosomal DNA (ITS rDNA) sequence (cox1 accession no./ITS accession no.: [a]GQ132130/GQ143711, [b]GQ132123/GQ143709, [c]GQ132122/GQ143709). H/BI: cox1 sequence data from anisakids infecting herring around the British Isles. Cross H/BI: cox1 sequence representing 153 anisakid individuals from herring around the British Isles (Cross et al. 2007). AY994157 *C. my*: *Anisakis simplex* cox1 from individual infecting *Conger myriaster* (Kim et al. 2006). *A. sim*: *A. simplex*, *A. peg*: *A. pegreffii*. Amino acids differing from consensus type are highlighted in black (RVS anisakids), dark grey (H/BI anisakids) and light grey (*A. pegreffii*)

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RVS cox1 C*ᵃ(5)V*ᵇ(4)   VMPTMIGGFG NWMLPLMLGA PDMSCPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
RVS cox1 C   GQ132133    VMPTMIGGFG NWMLPLMLGA PDMSCPRLNN LSFWLLLTAM FLILDSCFVD MGSGTSWTIY  60
RVS cox1 V   GQ132127    VMPTMIGGFG NWMLPLMLGA PDMSCPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
RVS cox1 V*ᶜ GQ132122    VMPTMIGGFG NWMLPLMLGA PDMSCPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60

A.sim DQ994401 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994375 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994333 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994391 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994356 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994353 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994243 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim DQ994325 H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60
A.sim Cross    H/BI      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60

A.sim AY994157 C.my      VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTIY  60

A.peg FJ907317           VMPTMIGGFG NWMLPLMLGA PDMSFPRLNN LSFWLLPTAM FLILDSCFVD MGSGTSWTVY  60


RVS cox1 C*ᵃ(5)V*ᵇ(4)   PPLSTMGHPG SSVDLAIFSL HSAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
RVS cox1 C   GQ132133    PPLSTMGHPG SSVDLAIFSL HSAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
RVS cox1 V   GQ132127    PPLSTMGHPG SSVDLAIFSL HSAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
RVS cox1 V*ᶜ GQ132122    PPLSTMGHPG SSVDLAIFSL HSAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120

A.sim DQ994401 H/BI      PPLSTMGHPG SSVDLAIFSL HCAGASSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994375 H/BI      PPLSTMGHPG SSVDLAIFSL HCAGASSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994333 H/BI      PPLSTMGHPG SSVDLAIFSL HCAGASSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994391 H/BI      PPLSTMGHPG SSVDLAVFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994356 H/BI      PPLSTMGHPG SSVDLAIFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994353 H/BI      PPLSTMGHPG SSVDLAIFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994243 H/BI      PSLSTMGHPG SSVDLAIFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim DQ994325 H/BI      PPLSTMGHPG SSVDLAIFSM HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120
A.sim Cross    H/BI      PPLSTMGHPG SSVDLAIFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120

A.sim AY994157 C.my      PPLSTMGHPG SSVDLAIFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120

A.peg FJ907317           PPLSTLGHPG SSVDLAIFSL HCAGVSSILG AINFMTTTKN LRSSSISLEH MSLFVWTVFV  120


RVS cox1 C*ᵃ(5)V*ᵇ(4)   TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
RVS cox1 C   GQ132133    TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
RVS cox1 V   GQ132127    TVFLLILSLP VLAGAITMLL TDLNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
RVS cox1 V*ᶜ GQ132122    TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPX  180

A.sim DQ994401 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994375 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994333 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994391 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994356 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994353 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994243 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim DQ994325 H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180
A.sim Cross    H/BI      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180

A.sim AY994157 C.my      TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGNPLI YQHLFWFFGH PEVYILILPA  180

A.peg FJ907317           TVFLLILSLP VLAGAITMLL TDRNLNTSFF DPSTGGIPLI YHHLF----- ----------  165


RVS cox1 C*ᵃ(5)V*ᵇ(4)   FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
RVS cox1 C   GQ132133    FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
RVS cox1 V   GQ132127    FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
RVS cox1 V*ᶜ GQ132122    FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240

A.sim DQ994401 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994375 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994333 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994391 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994356 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994353 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994243 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim DQ994325 H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240
A.sim Cross    H/BI      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240

A.sim AY994157 C.my      FGIISQSSLY LTGKKEVFGS LGMVYAILSI GLIGCVVWAH HMYTVGMDLD SRAYFTAATM  240

A.peg FJ907317           ---------- ---------- ---------- ---------- ---------- ----------  165


RVS cox1 C*ᵃ(5)V*ᵇ(4)   VIAVPTGVKV FSWLATLFGM KMVFQPX  267
RVS cox1 C   GQ132133    VIAVPTGVKV FSWLATLFGM KMVFQPX  267

RVS cox1 V   GQ132127    VIAVPTGVKV FSWLATLFGM KMVFQPX  267
RVS cox1 V*ᶜ GQ132122    VIAVPTGVKV FSWLATLFGM KMVFQPX  267

A.sim DQ994401 H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267
A.sim DQ994375 H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267
A.sim DQ994333 H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267
A.sim DQ994391 H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267
A.sim DQ994356 H/BI      VIAVPTGVKV FSWWATLFGM KMVFQPX  267
A.sim DQ994353 H/BI      VIAVPTGVKV FSWLATFFGM KMVFQPX  267
A.sim DQ994243 H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267
A.sim DQ994325 H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267
A.sim Cross    H/BI      VIAVPTGVKV FSWLATLFGM KMVFQPX  267

A.sim AY994157 C.my      VIAVPTGVKV FSWLATLFGM KMVFQPX  267

A.peg FJ907317           ---------- ---------- ------- 165
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(Fig. 1) and the fact that all analysed fish, irrespective of sample origin, showed consistent identical parasitological and pathological findings, suggest that all observed RVS-affected fish reported from different areas would reflect a similar condition.

Anisakis spp. in wild salmon is not an unusual finding, but is RVS a 'real emerging' condition or is it a newly detected condition (apparent emergence) due to location of the larvae and/or apparent higher prevalence in the host population? Major concerns were expressed by fishery managers and anglers regarding the impact of RVS on the fish and whether it could induce mortality or prevent spawning by any type of 'obstruction' of the genital area. Interestingly, the vent and urogenital papilla region rarely form part of any histopathological assessment, and this was reflected by the scarcity of bibliography thoroughly describing the region. Histological assessment of both male and female brood fish showed no evidence of obstruction or blocking of the genital cavity or pores with the parasites. Larvae could occasionally be seen within the lumen, but were likely to be migrating or pushed from the terminal portion of the abdominal cavity during stripping. However, larvae were responsible for some of the damage recorded in the vent and urogenital papilla of the analysed brood fish. Assisted spawning involves a certain degree of handling, including the compression of the vent when fish are manually stripped. Due to the pressure from the increased amount of cells and the 'masses of harder tissue' of encapsulated and non-encapsulated larvae in the area, tissue damage, including capillary breakdown, haemorrhage and a degree of inflammation, was observed. No material for 'comparative purposes' is available but a baseline level of tolerable and repairable damage is certainly expected after stripping. However, the most conclusive evidence of non-compromised spawning was a successful season of wild salmon under hatchery intervention in 2007.

An issue of public concern was whether RVS could affect or be linked to farmed Atlantic salmon. This syndrome was not reported in farmed fish and was restricted to wild Atlantic salmon. Previous studies have shown that anisakids are rare in farmed fish. In Japan, Norway and the USA, farmed salmonids (Atlantic salmon *Salmo salar*, coho salmon *Oncorhynchus kisutch*, Chinook salmon *O. tshawytscha* and rainbow trout *O. mykiss*) did not harbour *Anisakis* spp. in the viscera or muscle (Deardorff & Kent 1989, Inoue et al. 2000, Lunestad 2003), or the infection was very low (Inoue et al. 2000). Similarly, studies in Canada by Marty (2008) showed that by using *Anisakis* spp. prevalence in the viscera as an estimate of the parasite prevalence in the edible tissues, the risk ratio of *Anisakis* spp. in commercial farmed products was 570

times lower than in wild salmon. Despite extensive official surveillance, there are no reports of RVS-affected farmed salmon in Scotland; hence, the present study shows no direct link of aquaculture with RVS.

The reported prevalence of *Anisakis simplex* in wild Atlantic salmon varies from 65 to 100 % in the North, West and East Atlantic (Huss & Embarek 2003), while in the Puget Sound, North Pacific Ocean, 100 % of sockeye salmon *Oncorhynchus nerka* caught during their spawning migration were found to be infected (Deardorff & Kent 1989). However, a surprisingly limited amount of work has been carried out on *Anisakis* spp. infection in salmon compared to that on other commercially exploited fish such as Atlantic cod *Gadus morhua*, herring *Clupea harengus*, haddock *Melanogrammus aeglefinus*, mackerel *Scomber scombrus*, monkfish *Lophius piscatorius* and whiting *Merlangius merlangus*. Clear differences have been reported between the site location of anisakids species and their host and between host species (Smith 1984, Wootten & Jones 2007). Studies have shown that *Anisakis* spp. L3 tend to be more prevalent in the viscera or the musculature depending on the host's diet — more in the body cavity of euphausiid-feeding fish such as herring and mackerel but distributed throughout tissues among piscivorous hosts such as whiting and cod (Smith 1984). However, studies in Pacific chum salmon *O. keta* showed that 87 % of the larval load was recorded in the musculature (Deardorff & Kent 1989). Similarly, a study in the same species in Japan and the Bering Sea confirmed that *A. simplex* larvae were consistently more abundant in the skeletal muscle around the body cavity than in the visceral organs (Sugawara et al. 2004). These data seem to suggest that for salmonids at least, muscle infestation is more likely to occur than in other *Anisakis* spp. hosts. This is consistent with the present study data from RVS-affected wild Atlantic salmon, where a novel non-viscera location, the vent, and the very closely associated dorso-lateral musculature, proved to be able to harbour numerous larvae. To the best of our knowledge, other than for the previous preliminary reports for Scotland (Noguera et al. 2008) and recently, the report of RVS from England and Wales (Beck et al. 2008), *Anisakis* spp. have not been reported as being highly localised in a particular non-visceral region such as the vent, invading a discrete area at a high intensity. In the present study, although the data were qualitative and not non-quantitative, they showed no relationship between larval load in the body cavity and viscera, and the presence and numbers seen in the vent area. All analysed fish had low to moderate numbers of larvae in the body cavity but the numbers and extent of the lesion in the vent varied considerably from fish to fish. Moreover, it was observed in the present study that some of the fish

sampled as normal reference and negative fish collected later in the season were also positive for parasites in the vent area (data not shown). It seems unlikely that if present for as long as *Anisakis* spp. has been reported in the North Sea, RVS would have not been described. However, as the vent is frequently neglected during routine investigation, it is possible that the condition have been overlooked and under-reported until prominent lesions, such as the ones reported here, became conspicuous. This 'hyper-infestation' of the vent is therefore considered a novel site (as defined by Crompton 1973) for the parasite, and so far only reported for Atlantic salmon. The described observations and tissue changes in the vent-urogenital papilla constitute a particular type of lesion that can be considered pathognomonic, if not for the presence of the parasite, then for the condition described here as RVS.

Because of the novel site of infection and extent of tissue damage, it was hypothesised that the nematodes in this region might represent a different species of *Anisakis* from that normally found in the viscera and body cavity of wild Atlantic salmon. A report by Beck et al. (2008) associated the condition in England and Wales with *A. simplex* sensu lato, although the method for parasite identification was not reported. Molecular analysis in the present study based on ITS sequence identified the nematodes in the body cavity and vent as *A. simplex* s.s. based on previously defined diagnostic nucleotides and RFLP patterns. However, polymorphism was seen at a number of additional positions within some individuals analysed from both cavity and vent. Heterogeneous positions have been observed previously in the ITS rDNA of *Anisakis* spp. specimens, representing potential hybridisation events between *A. simplex* s.s. and *A. pegreffii*. The heterogeneity was observed at those sites diagnostic for each species. The heterogeneity was only observed in areas where sympatric coexistence occurred (Abollo et al. 2003, Farjallah et al. 2007). ITS sequences from anisakids isolated from Baltic Sea herring had a T at position 173, whereas other *A. simplex* s.s. possessed a C. These herring anisakids could represent the second 'variant' in a hybrid situation; however, they did not display the corresponding polymorphic nucleotides at the other positions observed in anisakids from RVS salmon. In addition, analysis of cloned ITS identified 3 different types of ITS variants, and not 2 as might be expected from a hybrid situation. The relative peak heights of the 2 nucleotides at the polymorphic sites did vary in amplitude between individuals (data not shown). It is worth noting that an anisakid sequence from onion-eye grenadier from east Greenland submitted to the NCBI GenBank sequence database in 2009 (Accession no. GQ131690) displayed an N at position

173 and a C/T polymorphism at 305, corresponding to the polymorphisms seen in some anisakids from RVS salmon. It is not clear from current sequence data whether the polymorphisms arose from hybridisation events (recent or distant) between different species, or between strains of *A. simplex*, or through intraspecific variation occurring naturally within the rDNA repeats. The level of variation in the ITS region can be very low even between defined *Anisakis* spp. (Nadler et al. 2005); therefore, even 2 nucleotide differences could indicate the emergence of a new *Anisakis* species/sub-species, differing in its life history. *cox1* mtDNA was also sequenced from nematodes found in cavity and vent. The *cox1* mtDNA is considered to evolve faster than rDNA (Blouin et al. 1998), but again, no clear differences in the *cox1* sequence were observed between the vent larvae and those from the body cavity, and between individuals with polymorphisms in their ITS rDNA. There is insufficient data to comment on whether the 2 conserved nucleotide/amino acid differences in the *cox1* sequence between anisakid specimens infecting RVS salmon and those infecting herring populations around the British Isles (Cross et al. 2007) is due to species or population differences. The latter individuals were collected from different hosts, with potentially different migration patterns and at a different time period.

While we had initially looked for differences between *Anisakis* spp. specimens infecting cavity and vent, it may be that the specimens characterised from RVS-affected salmon are variants which invade the cavity and vent rather than cavity alone, and as such, clear differences between sites of infection within a host may not be observed. More widespread sampling of anisakids from salmon with and without RVS, and/or infection experiments with different anisakid variants may provide an answer.

The mechanisms that determine the occurrence and distribution of *Anisakis* spp. in the northeast Atlantic ecosystem are largely unknown, but it has been proposed that *Anisakis* spp. in these waters occurs in fish from 2 ecologically different environments: the pelagic/oceanic and the benthic/coastal ecosystem (Levsen 2007). As anadromous fish, salmon are known to undertake extensive marine-feeding migrations (Mills 1991), and so they will inhabit both environments at different points during their sea life. Between the time smolts leave the river until mature fish return for spawning one or more years later, it is not known precisely when infestation of the vent takes place. However, preliminary observations from the present study showed different sizes of larvae observed macroscopically and histologically, some still migrating. Together with the observations of an acute inflammatory reaction in the early summer sam-

ples, this seem to indicate that the vent as a specific localisation had been affected during the last period when salmon would still be feeding. It should be stressed that the presence of different sizes of L3 as well as both encapsulated and non-encapsulated parasites could well reflect different times of ingestion and from a different prey, but as fish accumulate the parasite over time, it could also reflect the predation on other highly infected fish, resulting in the salmon acquiring a wide range of *Anisakis* spp. stages from a single meal. Histological assessment showed that the summer grilse had apparently more non-encapsulated larvae and signs of ongoing migration, reflecting a relatively recent infection. Conversely, analysis of brood fish in freshwater for several months showed a complete healing of external lesions and characteristic signs of tissue reaction such as granulomatous encapsulation and increased pigmented cell, differences likely reflecting time after settlement of larvae. Moreover, in a parallel study analysing the prevalence of the syndrome, it was noted that the following season (2008) showed a higher prevalence of moderate-to-severe RVS cases among the 2-sea-winter fish (C. Todd pers. comm.). This suggests that in the development of the syndrome, neither the mere fact of the presence of larvae (some obviously non-affected fish also harboured larvae in the vent), nor the stage at which salmon are infected are the most significant factors; what is most significant is the impact resulting from the cumulative effect of the high numbers of larvae within such a discrete volume of tissues (approx. 2 to 3 cm^3).

Fish collected in the sea or in estuarine waters (Strathy Point and North Esk) showed a strongly EGC-dominated inflammatory reaction; ECG is a type of cell considered to be analogous to the mast cells in helminth-infected mammals and birds (Reite 1997). The significance of this observation is not entirely clear, as it is possible that, rather than representing a specific reaction to *Anisakis* spp., they might reflect a physiological state of the fish. Persistent inflammation and massive recruitment of granular cells in the intestinal tissue of helminth-infested salmonids has been previously described (Murray 1972, Sharp et al. 1989, Ferguson 2006), but similarly, a massive degranulation of EGCs followed by an acute inflammatory reaction can be induced by the injection of hydrocortisone (Reite 1997). The histological analysis of samples from fish that had been in the freshwater for weeks or months showed that EGCs were not predominant anymore or were completely absent. Hence, the initial EGC-dominated inflammatory response, observed particularly in the early summer fish, may reflect at least partially the physiological stage of the fish (reproductive migration) rather than a specific reaction to the parasite.

While it was possible to hypothesise that a spontaneous change has occurred in *Anisakis* spp., it is more likely that the sudden North Atlantic-wide emergence of RVS could be a sign of ocean-scale changes in the environment, reflecting the North Atlantic occurrence of RVS. In host-parasite interactions, models and empirical studies of co-evolution assume that host resistance and parasitic infectivity are genetically based, though even in such cases non-genetic physiological or environmental influences can also alter host susceptibility (Dybdahl & Krist 2004). A factor valid at the ocean scale (and above), is water temperature, which can influence both parasite cycles and the host. Warming has been pronounced in the North Atlantic waters, with Scottish sea-surface temperatures rising 0.2 to 0.6°C per decade (Baxter et al. 2008), including in the foraging areas exploited by salmon (Todd et al. 2008). Seven of the 10 warmest years have occurred in the last decade, and 2006 was the second warmest year in UK coastal waters since records began in 1870 (Hughes 2006, Hughes & Holliday 2006, 2007, Hughes et al. 2008). Climate and climate variability affecting all components of the host-pathogen-environment triad have already been implicated as an important factor in the emergence of disease (Kutz et al. 2004). More generally, the 15°C summer isotherm has moved 330 km north in the last 17 yr (McMahon & Hays 2006), and studies by Beaugrand et al. (2008) in the North Atlantic showed that regions in which biome boundaries shifts occur are more vulnerable to the impact of climate change. There is a general tendency for parasitic infections to increase with temperature (Farjallah et al. 2006), and rising water temperatures have been reported to directly influence the incidence of disease threats from parasitic infections (Harvell et al. 1999, 2002, Hakalahti et al. 2006), particularly in temperate regions where transmission is strongly related to 'windows' of suitable temperature ranges. Increased infection pressure (as has apparently occurred with salmon) due to warming waters has been reported for metazoans (*Argulus coregoni* and *Diplostomum spathacum* usually found in fish culture) in Finland (Hakalahti et al. 2006). Infestation with *Anisakis* spp. of salmon or other fish predators may be particularly sensitive to temperature because infestation occurs via consumption of other infested intermediate hosts. There have been major changes in the distribution of plankton communities and fish species in the North Atlantic region (McMahon & Hays 2006), and recently biome boundaries movements due to climate warming have been suggested to possibly induce prey to follow alongside (Beaugrand et al. 2008). Another concomitant factor is fishing, although separating a response to climate variability from fishing practices has been difficult (Drinkwater et al. 2003).

Due to spatial and temporal differences, salmon might have been presented with reduced availability in prey and the need to compete (temporary absence of a given prey) or variability (different feeding options), leading to a shift in their diet, hence exposing them to increased *Anisakis* spp. loads. Interestingly, a decline in Atlantic salmon abundance has been reported for the last 3 decades, and warming seawaters of the northeast Atlantic have been associated with detrimental effects for this species. Todd et al. (2008) reported that both survival and growth have been impacted after analysis of 2 independent 14 yr time series, showing evidence of recent climate-driven changes in the eastern North Atlantic pelagic ecosystem. Finally, human allergic and gastro-allergic reactions to *A. simplex* were reported to have dramatically increased in Spain between 1995 and 2005 (Del Rey Moreno et al. 2006) and could reflect changes in culinary and feeding habits or improved diagnosis; however, it could also reflect, as reported by Valero et al. (2006), a real general increase in the prevalence of anisakid parasites in virtually all the wide range of fish hosts.

Unfortunately, establishing the specific implications of environmental changes on particular groups of interest will always be problematic, as historic data for comparative purposes are lacking. RVS might represent the observable final outcome of a chain of linked events where the increase in *Anisakis* spp. loads in salmon has only been highlighted by the conspicuous external lesions given the unique location of the parasite. The particular reasons for the dramatic increase in RVS observations in 2007 are as yet unclear; however, why the parasite is concentrated in the vent is a realistic area for further research. The consistent presence and parasite loads suggest the vent is unlikely to constitute an 'accidental location' and any advantage to the parasite on infesting the vent has yet to be elucidated.

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