DOI: 10.7589/2011-10-299 *Journal of Wildlife Diseases*, 49(2), 2013, pp. 229–234

RELATIONSHIP BETWEEN RED VENT SYNDROME AND ANISAKID LARVAE BURDEN IN WILD ATLANTIC SALMON (*SALMO SALAR*)

Sylvain Larrat,[1] Francis Bouchard,[2] Guylaine Séguin,[1,2] and Stéphane Lair[1,3]

[1] Canadian Cooperative Wildlife Health Center/Centre québécois sur la santé des animaux sauvages, Faculté de médecine vétérinaire, Université de Montréal, 3200, rue Sicotte, St. Hyacinthe, Quebec, Canada J2S 2M2
[2] Direction de l'expertise sur la faune et ses habitats, Ministère des Ressources naturelles et de la Faune. 880, Chemin Ste-Foy, 2e étage, Québec, Québec, Canada G1S 4X4
[3] Corresponding author (email: stephane.lair@umontreal.ca)

ABSTRACT: The pathogenesis of the recently recognized "red vent syndrome" in wild Atlantic salmon (*Salmo salar*) is not fully understood. Pathologic observations indicate that this syndrome is associated with the presence of nonencapsulated larvae of the nematode *Anisakis simplex* in the body wall, the lower intestinal wall, and the visceral cavity surrounding the vent region. We evaluated the relationship between the occurrence of red vent syndrome and intensity of infection with *Anisakis* sp. larvae in naturally infected fish. Salmon caught by sport anglers were opportunistically evaluated to detect red vent syndrome. We included 106 salmon with red vent syndrome and 98 without red vent syndrome in this study. Intensity of infection was established by counting the total number of perivisceral larvae and by determining the number of larvae per gram in 10 g of pepsin-digested perianal tissue. The severity of inflammatory changes was also evaluated in standard histologic sections of the perianal area using a semiquantitative scale. Salmon with red vent syndrome had significantly higher intensity of inflammation than salmon without red vent syndrome ($P=0.008$). The odds of having red vent syndrome increased with the number of perianal larvae per gram of perianal tissue ($P=0.002$; odds ratio [OR]=1.12; 95% confidence interval: [1.05; 1.22]) but not with the number of perivisceral larvae, fish length, or gender. Although these results support the association between this syndrome and intensity of infection by *A. simplex*, the relationship is not strong (OR near 1), suggesting that the clinical expression of red vent syndrome at an individual level, and the emergence of this disease on a global scale, must be determined by other factors, such as timing of infection.

Key words: *Anisakis simplex*, Atlantic salmon, intensity of infection, red vent syndrome, *Salmo salar*.

INTRODUCTION

The Atlantic salmon (*Salmo salar*), an emblematic fish of North Atlantic rivers, represents a significant resource for recreational and subsistence fishers. In the province of Quebec, Canada, wild Atlantic salmon populations are carefully managed through a comprehensive conservation program that includes stock monitoring by river, habitat preservation, regulation of recreational fishing based on a stock/recruitment model (Caron et al., 1999) and disease surveillance. As part of this surveillance program, red vent syndrome in spawning salmon was identified in the province of Quebec for the first time in 2008. This emerging syndrome of wild Atlantic salmon, which was first identified in Great Britain in 2004 (Beck et al., 2008), is characterized by reddening, swelling, and inflammation of the perianal region with or without cutaneous ulceration, scale loss, and bleeding. The lesions observed in this syndrome are associated with the presence of nonencapsulated nematode larvae of the species *Anisakis simplex* in the body wall, the lower intestinal wall, and the visceral cavity surrounding the vent region (Beck et al., 2008; Noguera et al., 2009). The adult nematodes are found in the gastric chambers of cetaceans. Marine fish can become paratenic hosts by eating the intermediate host, infected krill (Hays et al., 1998b). *Anisakis simplex* larvae can also become embedded in the gastric mucosa of humans when infected paratenic hosts are consumed raw or undercooked (Audicana and Kennedy, 2008). The reasons for the apparent emergence of red vent syndrome in salmon are not fully understood. A high intensity of parasitic larval

infection in salmon has been proposed as a determining factor in the development of this syndrome (Noguera et al., 2009).

We evaluated the relationship between red vent syndrome and intensity of infection by larvae of *Anisakis* sp. in naturally infected fish. If intensity of infection is a determining factor in this syndrome, salmon with red vent syndrome should present a higher intensity of infection than those without clinical signs.

MATERIAL AND METHODS

Sampling

Salmon from nine rivers managed by local associations were included in this study. These rivers (Bonaventure, York, Étamamiou, Aux Rochers, Petit Saguenay, Du Gouffre, Malbaie, St-Jean, and Matane) empty into the Estuary and Gulf of St. Lawrence (49°39′N, 66°15′W). The fish used for this study were captured prior to spawning by recreational anglers and brought to local registration stations. A technician performed an external examination and assessed whether the salmon showed perianal lesions consistent with red vent syndrome, particularly erythema and swelling of the perianal area, sometimes associated with hemorrhage, skin ulceration, and scale loss. Prior to the beginning of angling season, each of these technicians participated in training conducted by the principal investigator of this study (Larrat). Technicians were trained to recognize the lesions observed around the vent using photographs. For the purpose of this study, cases were defined as fish affected with red vent syndrome. The controls were salmon without visible lesions.

For each river, the technicians opportunistically selected up to 25 salmon with red vent syndrome and up to 15 control salmon, all measuring <63 cm in total length. This length restriction was dictated by provincial salmon fishing regulations aiming to discourage the capture of multi-sea-winter salmon over one-sea-winter salmon. In rivers in which keeping salmon ≥63 cm was allowed, up to 10 salmon ≥63 cm with red vent syndrome and 10 salmon ≥63 cm without red vent syndrome were also selected. For selected fish, all coelomic viscera and the abdominal wall surrounding the vent were sampled and conserved frozen in individual plastic bags for analysis at the end of the fishing season.

Larval counts and histology

The samples were thawed at room temperature. The tissues were gently dissected to enable full counts of *Anisakis* sp. larvae on the serosal aspect of the coelomic viscera. Each perianal region was cut in two equal parts following the sagittal plane. A maximum of 10 g of the left perianal region was digested in a mixture of hydrochloric acid (Fisher Scientific, Whitby, Ontario, Canada; 30 ml/g of fish) and pepsin (Fisher Scientific; 300 mg/g of fish) at 40 C (method adapted from Stern et al., 1958). The digestion lasted until the flesh was completely dissolved. The liquid obtained was then filtered in a 150-μm sieve to recover and count *Anisakis* sp. larvae. Fragments of larvae were counted only if they included the cranial extremity of the nematode. The right side of the perianal area was trimmed following a parasagittal plane. The medial section was fixed in 10% buffered formalin, embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin-phloxine-saffron (Luna, 1968). Each histologic section was examined by light microscopy to record the presence or absence of *Anisakis* sp. larvae and to evaluate intensity of inflammation, using the following scale: grade 0=no inflammation or scattered individual inflammatory cells; grade 1=mild inflammatory infiltrates; grade 2=moderate inflammatory infiltrates localized only around nematode larvae; and grade 3=marked coalescing to diffuse inflammatory infiltrates. All evaluations were performed by observers using single-blind method regarding clinical expression of red vent syndrome.

Statistical analysis

A standardized index of perianal infection intensity, referred to hereafter as the number of perianal *Anisakis* sp. larvae per gram, was obtained by dividing the number of perianal larvae recovered from digestion by the weight of the sample. The software R (R Foundation for Statistical Computing, Vienna, Austria) was used to perform logistic regressions, McNemar chi-square tests, and chi-square tests of independence. The effects of the number of visceral *Anisakis* sp. larvae, the number of perianal *Anisakis* sp. larvae per gram, salmon length, and salmon gender on the presence or absence of macroscopic lesions were evaluated. Gender was included in the variables tested because individuals of one gender, usually males, are sometimes more heavily parasitized (Poulin, 1996). Length was also included because larger individuals may have consumed more paratenic hosts than smaller individuals and may therefore have a higher

parasite burden (Lo et al., 1998). Because weight was strongly correlated to length (Spearman's rank correlation rho: 0.9, $P<2e-16$), weight was not included in the analysis.

Each variable was first tested individually. The variables associated with a P value <0.2 were included in the full model. The parsimony model was chosen with a backward stepwise variable elimination algorithm based on the P values (Dohoo et al., 2003). Results were considered statistically significant when P values were <0.05.

RESULTS

Larval counts

Viscera from 204 salmon were analyzed: 106 with red vent syndrome and 98 without. Sufficient perianal tissue was available in 194 of these fish: 105 with red vent syndrome and 89 without. *Anisakis* sp. larvae were present on the visceral surface of 200/204 salmon and in the perianal region of 170/194. Only 1/194 fish had no larvae on the viscera and digested vent area; however, there were sections of nematode larvae in its perianal histologic sections. The number of *Anisakis* sp. larvae counted from the serosal aspect of the coelomic viscera ranged from 0 to 198 (median: 20). The number of perianal larvae per gram of perianal tissue ranged from 0 to 28 (median: 2.5). The median numbers of visceral larvae in salmon with red vent syndrome and in salmon without external clinical signs were 30.5 and 16.5 larvae, respectively. The median numbers of larvae per gram in salmon with red vent syndrome and in control salmon were 3.5 and 1.7 larvae per gram, respectively (Fig. 1). The number of perianal larvae per gram was the only variable significantly associated with the presence of red vent syndrome (logistic regression, $P=0.002$; odds ratio [OR]= 1.12; 95%; confidence interval for OR: [1.05; 1.22]). The number of visceral larvae ($P=0.49$), length ($P=0.55$), and gender ($P=0.98$) of salmon had no significant effect on the presence of red vent syndrome.

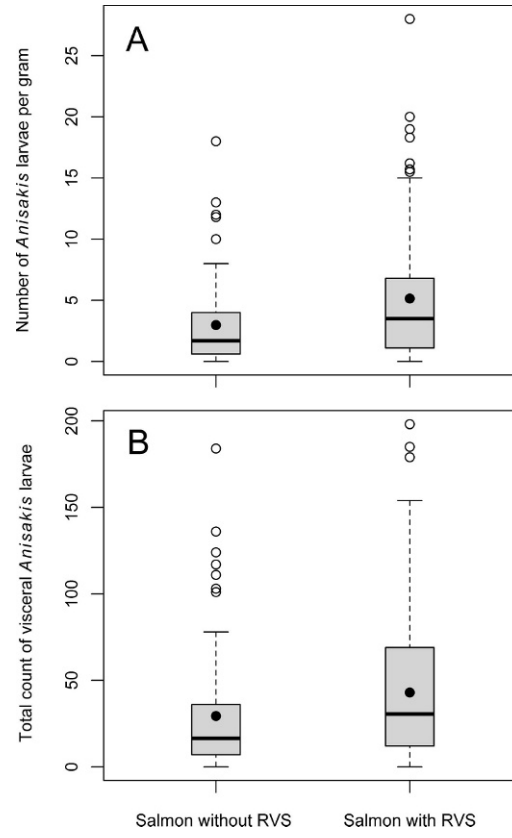

FIGURE 1. Box plots presenting the distribution of the number of the nematode *Anisakis* sp. larvae per gram in the vent region (A) and of total larval count on the serosal aspect of the coelomic viscera (B) in Atlantic salmon (*Salmo salar*) with and without red vent syndrome (RVS). Horizontal lines represent the medians. Black dots correspond to the means. Grey boxes encompass the second and third quartiles. The whiskers represent the highest and lowest data included in 1.5 times the 25–75% percentile. The white dots are outliers. Although salmon with red vent syndrome generally present a slightly higher parasite load, there is no obvious segregation between the two groups.

Histology

Histologic slides were available for 196 salmon: 106 with red vent syndrome and 90 without. At least one larval section was present in 166/196 salmon. No significant difference was noted in presence of larvae in fish with and without red vent syndrome (Table 1; chi-square test of independence, $P=0.19$). Digestion and histologic examinations significantly differed in

TABLE 1. Distribution of salmon (*Salmo salar*) from the Province of Quebec, Canada, according to the presence or absence of at least one larval section of the nematode *Anisakis* sp. in histologic sections of the vent region in relation to the presence or absence of red vent syndrome (RVS). No statistically significant difference was detected between the two fish groups (chi-square tests of independence, $P=0.19$).

	Detection of larvae in histologic evaluation	
	No	Yes
Salmon without RVS ($n=90$)	17	73
Salmon with RVS ($n=106$)	13	93

their ability to assess presence or absence of larvae (McNemar chi-square test, $P=0.003$). Histologic examination failed to identify nematode larvae in 23 perianal regions from which larvae were found by digestion. Conversely, six salmon from which no larvae were found by digestion had nematode sections visible histologically. The total number of salmon with larvae in their perianal regions was thus 176/194.

Intensity of inflammation was significantly higher in salmon with red vent syndrome than in salmon without red vent syndrome (Table 2; chi-square test of independence, $P=0.008$). No larvae were present in the slide graded 0 (no inflammation). Inflammation was most consistently surrounding the nematodes visible on the tissue sections. The inflammatory infiltrates consisted in increasing amount of macrophages with increasing grade. In addition, melanomacrophages, multinucleated giant cells, and eosinophilic granular cells were present in grade 3 infiltrates.

TABLE 2. Grading of inflammatory changes evaluated in histologic sections of salmon (*Salmo salar*) from the Province of Quebec, Canada, with and without red vent syndrome (RVS). Salmon with RVS showed significantly higher intensity of inflammation (chi-square test of independence, $P=0.008$).

	Grading of inflammation in vent region[a]			
	0	1	2	3
Salmon without RVS ($n=90$)	8	16	36	30
Salmon with RVS ($n=106$)	8	7	27	64

[a] Grade 0 = no inflammation or scattered individual inflammatory cells; grade 1 = mild inflammatory infiltrates; grade 2 = moderate inflammatory infiltrates localized around nematode larvae; grade 3 = marked coalescing to diffuse inflammatory infiltrates.

DISCUSSION

Larvae of *Anisakis* sp. were present in the perianal region of 91% of examined salmon, regardless of the presence or absence of red vent syndrome. This confirms the observation by Noguera et al. (2009) that only the presence of *Anisakis* sp. larvae is not sufficient to explain the macroscopic lesions observed in salmon with red vent syndrome. The differences in intensities of inflammation observed microscopically paralleled the macroscopic lesions; salmon with red vent syndrome generally had higher grades of inflammation than control salmon. Although fine-scale evaluation of the histologic sections was limited by changes due to freezing, inflammatory infiltrates were located around nematode larvae, as reported previously (Beck et al., 2008; Noguera et al., 2009). Histologic examination of the perianal region was less sensitive than enzymatic digestion in assessing the presence of *Anisakis* sp. larvae. Digestion of the circumferential perianal region may provide optimal sensitivity, especially in fish with very low intensity of infection, in which larvae may be asymmetrically distributed.

Potential biases in the current study might have resulted from the rejection of most severely diseased salmon by anglers or from misclassification of cases and controls by technicians. Training and reference photographs were used to limit the later scenario. The extent to which anglers preferentially select healthy-looking salmon is not known but believed to be low. In addition, *Anisakis* larvae are known to migrate from viscera to muscles after the death of their hosts (Smith

and Wootten, 1975). Although variations in the time from death to sampling could have caused a bias, this variable was assumed to be evenly distributed between groups of salmon with or without lesions.

Noguera et al. (2009) proposed that the cumulative effect of a large number of larvae concentrated in the vent region might be a determining factor in the occurrence of this syndrome. Our study supports this hypothesis by showing that the odds of having red vent syndrome significantly increased with the number of larvae per gram detected in perianal tissue. Consequently, this finding suggests that the apparent emergence of red vent syndrome might, at least partially, be attributed to an overall increase in *Anisakis* sp. burden of Atlantic salmon over the past few years. If proven true, such an increase in intensity of infection would suggest a higher exposure of salmon to this parasite through their diet. Atlantic salmon eat fish and various species of crustaceans, such as krill (Jacobsen and Hansen, 2001). *Anisakis* sp. larvae are more abundant in paratenic hosts, such as capelin and herring, than in the intermediate host, krill (Hays et al., 1998a, b). A decrease in krill abundance and an increase in capelin and herring stocks have been reported over the last decade in the Gulf of St. Lawrence (Dufour et al., 2010). A shift in abundance of salmon's prey, with an increase in paratenic hosts, might account for an increased exposure to *Anisakis* sp. larvae.

However, the relationship between the number of perianal larvae and the presence of macroscopic lesions is not believed to be biologically strong, because an OR equal to 1 would represent an absence of effect and the OR obtained was 1.12. The weakness of this relationship is also revealed by the lack of graphic segregation between salmon with and salmon without red vent syndrome in relation to the number of larvae per gram of perianal tissue (Fig. 1). This observation indicates that larval count is not the only determining factor in red vent syndrome. In addition, even though *A. simplex* has been reported to parasitize wild salmon for over 100 yr (Carmichael, 1863), it was not previously associated with red vent syndrome (Beck et al., 2008; Noguera et al., 2009). Consequently, the presence of red vent syndrome at an individual level and the emergence of this disease on a global scale must be determined by other factors.

Noguera et al. (2009) hypothesized that the emergence of this syndrome might be caused by *A. simplex* strains of higher pathogenicity. However, this hypothesis has not been validated. Similarly, the presence of lesions could be affected by variations in factors impacting the immune system of fish, such as genetic variations, reproductive status, sex, temperature of water, or stress (Magnadottir, 2010).

Timing of infection and stage of pathogenesis of disease may also play an important role in the presence of lesions, when salmon enter rivers where they are sampled. It is reasonable to assume that in salmon with acute infections of *Anisakis* sp., perianal larvae migrations could elicit active inflammation, causing congestion, edema, hemorrhage, and swelling around the vent. Conversely, in chronically infected fish, the larvae would have stopped their migration and remained encapsulated in the perianal tissues. These chronically infected fish would not be classified as cases of red vent syndrome because the acute lesions would have healed at the time of sampling. This proposed healing mechanism is supported by observations made on affected fish held in captive settings. Indeed, active lesions of red vent syndrome in naturally affected salmon kept in a provincial hatchery have healed within 6 mo, even though nematode larvae surrounded by chronic granulomatous reactions were still present (Serge Guimond, *Ministère des Ressources naturelles et de la Faune*, pers. comm.). Accordingly, the apparent emergence of red vent syndrome in salmon returning to rivers could be, at least in part, associated with an increase in the proportion of salmon becoming infected at a later stage of their journey in the marine environment.

Our results support the possible relationship between the presence of red vent syndrome in Atlantic salmon and a high intensity of infection by *Anisakis* sp. but also suggest that there might be other determining factors, such as timing of infection. Additional studies are needed to better understand the pathogenesis and significance of red vent syndrome for Atlantic salmon populations.

ACKNOWLEDGMENTS

We thank the river management associations and the *Ministère des Ressources naturelles et de la Faune du Québec*, who provided an essential support to the study. We also thank the staff of the *Centre québécois sur la santé des animaux sauvages* for technical assistance. This study was partly funded by the *Ministère des Ressources naturelles et de la Faune du Québec* and the *Fond du centenaire de l'Université de Montréal.*

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Submitted for publication 11 October 2011.
Accepted 4 March 2012.