



Host size influences prevalence and severity of *Kudoa thyrsites* (Cnidaria: Myxosporea) infection in Atlantic salmon *Salmo salar*

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ABSTRACT: *Kudoa thyrsites* is a cosmopolitan myxozoan parasite of marine fish. The infection causes an economically important myoliquefaction in farmed Atlantic salmon in British Columbia, Canada. Laboratory exposure of Atlantic salmon smolts to infectious seawater was used to test the hypothesis that infection with *K. thyrsites* is more severe in age-matched, smaller salmon. In each of 2 trials approximately 4 mo apart, smolts were graded into small (80 and 68 g), medium (117 and 100 g) and large (142 and 157 g) initial weight groups (IWGs) and concurrently exposed to infectious seawater. The effects of IWG and time on fish size and infection severity were assessed by linear mixed-effects models. The fish were screened for infection by histological examination at intervals following exposure. Increases in mean length and weight were statistically significant in all IWG during both trials. The infection was detected in fish in both trials, and in Trial 2, the prevalence was significantly greater in larger fish 1000 degree-days (DD) after exposure. The severity of infection (plasmodia mm⁻² muscle) was significantly higher in larger smolts: between medium and large IWGs at 2500 DD in Trial 1 and between small and medium IWGs at 1500 and 2000 DD in Trial 2. The hypothesis is rejected and possible explanations for the greater occurrence of *K. thyrsites* in larger smolts are discussed.

KEY WORDS: *Kudoa thyrsites* · Myxozoa · Atlantic salmon · *Salmo salar* · Size · Muscle · Infection

1. INTRODUCTION

Kudoa thyrsites is a myxozoan parasite of the muscle of marine fishes. In the Pacific waters of North America, the Pacific hake *Merluccius productus* is an important host although *K. thyrsites* has also been reported from numerous other fish species (Kabata & Whitaker 1981, Kabata & Whitaker 1989, Moran et al. 1999a, King et al. 2012). In most hosts, parasitic plasmodia containing the characteristic stellate myxospores develop within myocytes of the striated muscle, although in Pacific salmon *Oncorhynchus* spp. and infrequently in Atlantic salmon *Salmo salar*, the parasite also occurs within cardiac myocytes (Kabata

et al. 1986, Kabata & Whitaker 1989, Moran et al. 1999b). Harrell & Scott (1985) first reported *K. thyrsites* in seawater netpen-reared Atlantic salmon in Puget Sound, Washington, USA, and the infections have since been well documented in cultured Atlantic salmon in British Columbia (BC), Canada (St Hilaire et al. 1997, Marshall et al. 2016). Fish are exposed to the parasite in infectious seawater that is assumed to contain the infective stage, likely an actinospore. Plasmodia require several weeks to months to develop. Despite the ubiquitous distribution and broad host range of the parasite, the life cycle has not been elucidated and an invertebrate host has not been described.

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In Atlantic salmon, post-mortem myoliquefaction at the site of *K. thyrsites* infection reduces fillet quality, with a significant cost to industry (Funk et al. 2008, Lafferty et al. 2015, Jones et al. 2016). The severity of tissue degradation is proportional to the parasite burden in the muscle (Dawson-Coates et al. 2003, Funk et al. 2007) and is caused in part by proteolytic enzymes, including a cathepsin L that is secreted by the plasmodium (Funk et al. 2008).

Although clinical signs are not associated with the infection, there are host-specific patterns of parasite development and host response. In Pacific hake, there is no evidence of a host response prior to complete occupation of the myocyte by the parasite (Stehr & Whitaker 1986). During co-infections with *K. thyrsites* and *K. paniformis*, affected muscle cells become encapsulated and eventually heavily pigmented, and this process occurs more frequently in older fish (Morado & Sparks 1986). In Atlantic salmon, the infection is self-limiting (Moran & Kent 1999, Moran et al. 1999b, Jones et al. 2016) and recovered fish are refractory to reinfection (Jones et al. 2016). Infiltration of infected myocytes with inflammatory cells was initially believed to coincide with plasmodium maturation and rupture, a process that resulted in destruction of the myocyte (Moran et al. 1999b). Cells within this inflammatory lesion are known to phagocytise myxospores (Moran et al. 1999b). More recent observations indicate that cellular infiltration occurs shortly after infection of the myocyte (Braden et al. 2018), indicating that host recognition of the infection occurs at an early stage. Cells in this early infiltrate stage bear markers consistent with antigen-presenting macrophages, dendritic cells and/or cytotoxic T-cells. Together, these data are consistent with a protective immune response to *K. thyrsites* in Atlantic salmon, the initiation of which occurs during early parasite development within the myocyte.

The development of severe *K. thyrsites* infections in Atlantic salmon cultured in BC is mitigated by the practice of indirect entry: temporary rearing of smolts at production sites with a low *K. thyrsites* exposure risk, prior to grow-out at high-risk sites. While it is possible that prior exposure to the parasite at low-risk sites elicits an acquired immune response, an alternative hypothesis is that the lower susceptibility of salmon upon transfer to the high-risk site is related to the increased size of the salmon at that time. The present study tests the hypothesis that risk of infection with *K. thyrsites* is increased in smaller post-smolt Atlantic salmon.

2. MATERIALS AND METHODS

2.1. Exposure to *Kudoa thyrsites* and sample collection

Two experimental trials were conducted at the Pacific Biological Station located at Departure Bay, Nanaimo, BC, Canada. Both trials involved exposure to raw seawater from Departure Bay, which leads to infection with *K. thyrsites* in naïve Atlantic salmon (Moran et al. 1999b, Jones et al. 2012, 2016). Naïve Atlantic salmon smolts were obtained in the autumn of 2015 (Trial 1) and the spring of 2016 (Trial 2) from 2 commercial hatcheries on Vancouver Island. Those obtained in 2015 were graded into initial weight groups (IWGs) of approximately 80, 117 and 142 g, while those obtained in the spring of 2016 were graded into IWGs of approximately 68, 100 and 157 g. The trials were designed to test the effect of host size on susceptibility to *K. thyrsites*, and in Trials 1 and 2, 200 fish in each IWG were assigned to each of two 2500 l tanks and acclimated to ultraviolet-irradiated seawater (UVSW) over periods of 63 and 32 d, respectively. Fish were fed a pelleted ration (Ewos Canada) at a daily rate of 1% of tank biomass. Exposure to the parasite was facilitated by replacement of UVSW with raw seawater supplied to the experimental tanks as reported earlier (Jones et al. 2016). In Trial 1, UVSW was replaced between 4 January and 27 February 2016, for an exposure of 512 degree-days (DD). In Trial 2, UVSW was replaced between 9 May and 23 June 2016, for an exposure of 522 DD. In both trials, the flow of UVSW was resumed thereafter and onset of exposure and accumulation of DD coincided with the first day of UVSW replacement. See Jones et al. (2016) for complete details of fish husbandry, exposure and sampling, which followed guidelines of the Canadian Council of Animal Care (Pacific Region Animal Care Committee AUP 15-009).

In Trials 1 and 2, samples were collected at 1093 and 1038 DD, 1588 and 1557 DD, 2097 and 1983 DD, and 2617 and 2583 DD, respectively. For clarity, these sampling times are herein identified as 1000, 1500, 2000 and 2500 DD. Immediately prior to sampling, the fish were sedated by adding 25 mg l⁻¹ Aquacalm (Syndel Laboratories, Canada) to the tank water, then killed in seawater containing 250 mg l⁻¹ MS-222 (Syndel). Length and weight were measured for each fish. Three pieces of epaxial skeletal muscle were dissected from standardized locations anterior to, adjacent to and posterior to the dorsal fin and preserved in neutral buffered 10% formalin (NBF). In Trial 1, samples of cardiac muscle were also collected

at 2000 and 2500 DD and preserved in NBF. After 5 to 7 d, the NBF was replaced with 70% isopropanol for sample storage at room temperature.

2.2. Histology and assessment of infection

In both trials, NBF-fixed muscle samples from up to 20 fish per tank were dehydrated in an alcohol gradient, clarified in xylene and infiltrated with paraffin wax for subsequent sectioning, staining with hematoxylin and eosin, and histological examination (see Jones et al. 2012). An infection severity index for each fish was calculated as the arithmetic mean number of *K. thyrsites* plasmodia per mm² from the 3 muscle sections. The median infection severity for each IWG was estimated from the severity indices of individual fish.

2.3. Statistical analyses

The significance of differences in the proportions of fish with detectable infections by IWG and time was determined using Chi-square tests. Statistical significance was assumed when $p < 0.05$. In each trial, the effects of IWG and time on length, weight and infection severity were analyzed with linear mixed-effects (LME) models using the nlme package in R version 3.4.3 (Pinheiro et al. 2017, R Core Team 2017). The fixed-effect terms were DD post exposure, IWG groupings and their interaction. The random effect was tank, and a term for unequal variance between treatments was included. For post hoc analyses, least-square means were generated in R using lsmeans from the package emmeans (Lenth 2018) with the lme model with the interac-

tion term. Mean values were compared pairwise and adjusted p-values (Tukey) determined statistical significance.

3. RESULTS

From onset of exposure to final sampling, the durations of Trials 1 and 2 were 241 and 219 d, respectively. In Trial 1, the median (range) temperature during exposure to infectious seawater was 9.3°C (9.1–10.2°C) and median salinity was 29.6 ppt (28.9–30.3 ppt). In Trial 2, these values were 11.3°C (10.5–12.7°C) and 29.4 ppt (28.2–29.8 ppt), respectively. The differences in median temperature ($p < 0.001$, Mann-Whitney) and salinity ($p = 0.044$, Mann-Whitney) between the 2 exposures were statistically significant.

3.1. Fish growth

Length and weight of experimental salmon increased in all IWGs over the course of both trials (Table 1). Length and weight were significantly influenced by the main effects (IWG, sample time) and in neither trial was the interaction between the two significant (see Tables S1 & S2 in the Supplement at www.int-res.com/articles/suppl/d133p099_supp.pdf). In Trial 1, comparisons of length and weight among all IWGs were statistically significant (Tables S1 & S2). Similarly, all comparisons among sample times but one (1500 versus 2000 DD) were significant. In Trial 2, all comparisons of length and weight among sample times were statistically significant ($p < 0.0001$), as were comparisons among IWGs ($p < 0.0001$; Tables S4 & S5 in the Supplement).

Table 1. Mean (\pm SD) length and weight of Atlantic salmon *Salmo salar* by initial weight group (small, medium or large) at intervals following exposure to *Kudoa thyrsites* infective seawater in Trials 1 and 2 (see Section 2.1 for details)

Time (degree-days)	Fork length (cm)			Weight (g)		
	Small	Medium	Large	Small	Medium	Large
Trial 1						
1000	26.8 \pm 4.5	31.5 \pm 3.1	31.5 \pm 2.6	228.7 \pm 115.1	349.8 \pm 90.9	351.5 \pm 91.0
1500	28.7 \pm 3.8	32.0 \pm 3.5	33.0 \pm 3.9	253.3 \pm 107.2	355.1 \pm 125.4	379.1 \pm 146.0
2000	30.1 \pm 3.7	33.7 \pm 3.6	35.0 \pm 3.8	274.1 \pm 104.4	396.7 \pm 150.0	448.6 \pm 173.4
2500	33.2 \pm 3.1	36.6 \pm 3.4	37.4 \pm 3.1	380.4 \pm 110.6	539.5 \pm 155.0	576.5 \pm 165.6
Trial 2						
1000	26.0 \pm 1.7	28.9 \pm 1.9	30.1 \pm 2.3	171.1 \pm 38.3	237.0 \pm 47.8	279.3 \pm 77.4
1500	28.0 \pm 2.1	30.5 \pm 2.3	32.2 \pm 2.5	202.8 \pm 47.3	275.9 \pm 65.2	337.7 \pm 73.2
2000	30.4 \pm 1.6	33.2 \pm 2.6	35.4 \pm 2.8	295.9 \pm 47.1	387.4 \pm 81.2	493.6 \pm 116.0
2500	34.3 \pm 2.4	37.4 \pm 3.1	40.8 \pm 2.8	435.8 \pm 81.2	560.4 \pm 128.8	757.6 \pm 154.9

3.2. Infection with *Kudoa thyrsites*

In Trial 1, *Kudoa thyrsites* was undetected in salmon from the small and medium IWGs sampled at 1000 DD (Table 2). In all other IWG–DD combinations, the prevalence ranged from 4.2% in large IWG salmon at 1000 DD to 42.5% in medium IWG salmon at 2500 DD. Differences in prevalence among sample times were statistically significant only in the medium IWG, and there were no statistically significant differences in prevalence among IWGs at any sample time (Table 2). Regarding infection severity (plasmodia mm⁻²), the interaction of IWG and sample time was statistically significant ($p = 0.032$; Table S3 in the Supplement). At 2500 DD, infection severity was significantly different ($p = 0.003$) between the medium and large IWGs (Table S3, Fig. 1). There was no evidence of the infection in heart muscle of 78 salmon collected from the large IWG at 2000 DD ($n = 39$) and 2500 DD ($n = 39$).

In Trial 2, the parasite was detected in all IWG–DD combinations. The prevalence ranged from 26.3% in salmon from the small IWG at 1000 DD to 100% in salmon from the large IWG at 1500 DD and the small IWG at 2000 DD (Table 2). Differences in prevalence among sample times were statistically significant in the small IWG, and among IWGs they were significant at 1000 DD. There was a statistically significant ($p < 0.029$) interaction between IWG and sample time (Table S6 in the Supplement). At both 1500 and

2000 DD, the difference in infection severity between the small and medium IWGs was significantly different (Table S6, Fig. 2).

4. DISCUSSION

In this study, we showed that naïve Atlantic salmon acquired infections with *Kudoa thyrsites* following relatively brief exposures to raw seawater from Departure Bay, BC, Canada, confirming our earlier observations (Jones et al. 2012, 2016). The effect of host size on the outcome of the *K. thyrsites* infection was examined under controlled laboratory conditions. Furthermore, LME models of host length and weight in the age-matched salmon confirmed that the IWGs were distinct size categories and maintained throughout the study, suggesting that on average, fish in the different IWGs differed in growth rates. This is not the first study to examine the effect of host size on the outcome of exposure to *K. thyrsites* infectious seawater. Over an 8-mo period, Jones et al. (2016) found evidence for increased risk of *K. thyrsites* in smaller salmon smolts within a population that had been serially exposed over time. As acknowledged in the earlier study, however, this apparent size effect was confounded by an uncontrolled seasonal effect in which the smaller fish were exposed during summer months. Of the 2 trials reported here, seawater during the late spring exposure (Trial 2) was warmer but less saline than that in the early winter (Trial 1). Previously, infections at this location were acquired by naïve Atlantic salmon during summer and autumn exposures but not during those in winter and early spring (Moran & Kent 1999). Similarly, the abundance of *K. thyrsites* DNA is higher in seawater samples collected in late summer to autumn compared to those collected in winter (Jones et al. 2016), suggesting an increased infection pressure in late summer and autumn. In addition, because the salmon used in each trial were derived from different salmon production companies, genetic influences on the unequal magnitudes of infection observed in the trials should not be ruled out. The key finding from the present study was that despite the seasonal differences

Table 2. Effect of size (see Section 2.1 for definition of initial weight group) and time post-exposure on prevalence of *Kudoa thyrsites* in skeletal muscle samples (Prev. = number positive/number examined \times 100%; by microscopic examination) from Atlantic salmon *Salmo salar*. Salmon in both trials were exposed to infectious seawater for approximately 520 degree-days (see Section 2.1)

Time (degree- days)	Initial weight group						χ^2 (p-value)
	Small		Medium		Large		
	n	Prev.	n	Prev.	n	Prev.	
Trial 1							
1000	24	0.0	24	0.0	24	4.2	1.95 (0.378)
1500	40	12.5	40	25.0	39	20.5	1.414 (0.493)
2000	40	15.0	40	20.0	40	32.5	2.304 (0.316)
2500	40	17.5	40	42.5	40	40.0	3.649 (0.161)
χ^2	3.855		9.917		7.138		
(p-value)	(0.278)		(0.019)		(0.068)		
Trial 2							
1000	38	26.3	39	61.5	38	81.6	7.288 (0.026)
1500	40	90.0	40	97.5	40	100.0	0.117 (0.943)
2000	40	100.0	40	97.5	40	95.0	0.026 (0.987)
2500	40	87.5	40	80.0	39	94.9	0.267 (0.875)
χ^2	12.185		2.370		0.415		
(p-value)	(0.007)		(0.499)		(0.937)		

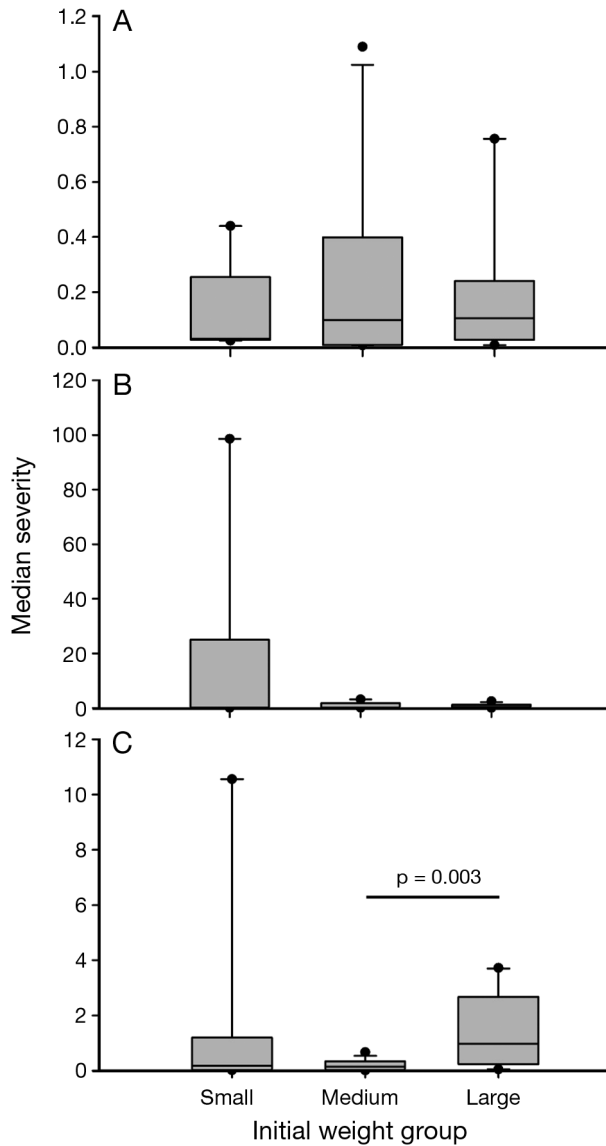


Fig. 1. Trial 1: Initial weight group (IWG)-associated median severity (plasmodia mm^{-2}) of *Kudoa thyr sites* in Atlantic salmon *Salmo salar* skeletal muscle at (A) 1500, (B) 2000 and (C) 2500 degree-days following exposure to infectious seawater (note y-axis scales differ among plots; see Section 2.1 for IWG definitions). Boxes: median and 25th/75th percentiles; whiskers: 10th/90th percentiles; dots: 5th/95th percentiles. Significant differences between treatments at a given sampling time are denoted by p-values

between trials and possible genetic effects, similar patterns were observed such that infections in larger salmon had a shorter prepatent period and tended to be more prevalent and more severe than in the smaller fish.

The current data indicate that exposure of larger salmon resulted in infections with a more rapid onset and greater severity than concurrent exposure of

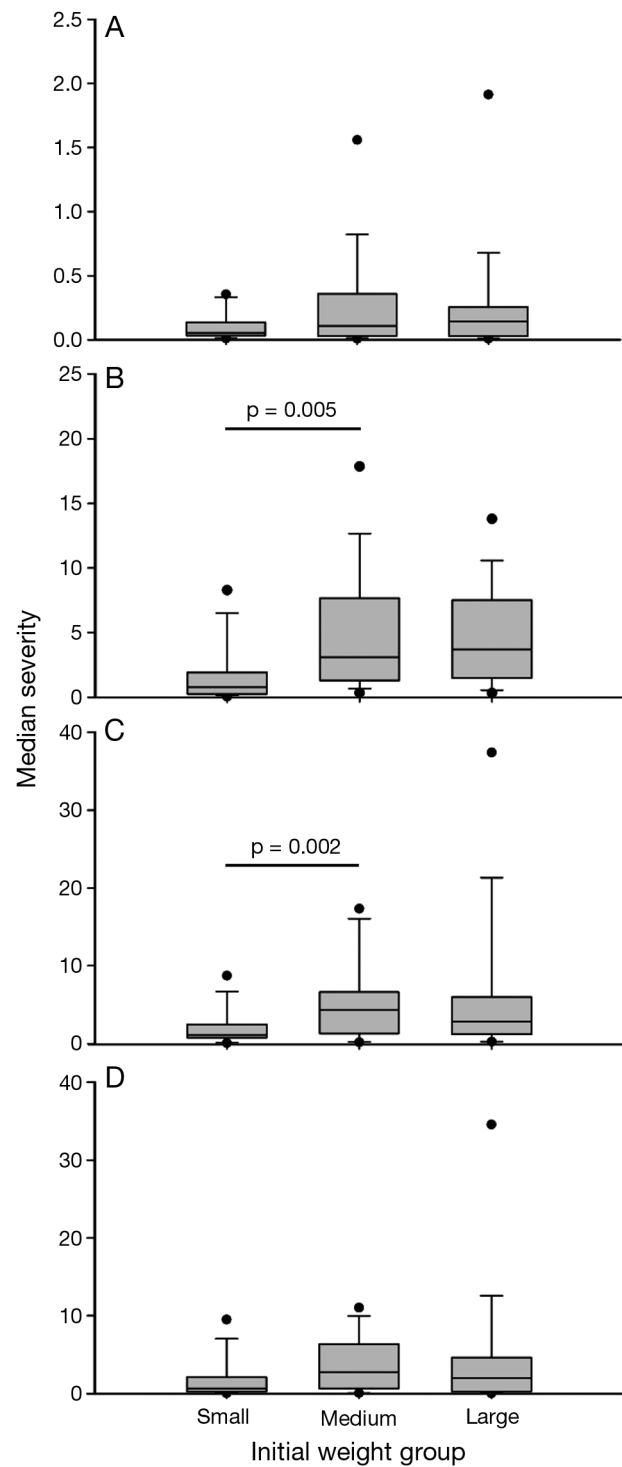


Fig. 2. Trial 2: Initial weight group (IWG)-associated median severity (plasmodia mm^{-2}) of *Kudoa thyr sites* in Atlantic salmon *Salmo salar* skeletal muscle at (A) 1000, (B) 1500, (C) 2000 and (D) 2500 degree-days following exposure to infectious seawater (note y-axis scales differ among plots; see Section 2.1 for IWG definitions). Boxes: median and 25th/75th percentiles, whiskers: 10th/90th percentiles, dots: 5th/95th percentiles. Significant differences between treatments at a given sampling time are denoted by p-values

smaller fish. Speculation on possible explanations for this size-associated pattern of infection may be helpful in defining future research. It has been established that the skin, gills and buccal cavity are routes of infection for several myxozoan parasites (Kallert et al. 2007). Additionally, in freshwater teleosts, gill surface area increases allometrically with fish weight (Palzenberger & Pohla 1992), and the surface area of juvenile Atlantic salmon (exclusive of gills) varies directly with length or weight (Tucker et al. 2002). A significant positive relationship was reported between swimming performance and spore density in *K. inornata*-infected sea trout (McElroy et al. 2015). Those authors rejected the hypothesis that this relationship reflects a tendency for more severe infection in larger fish, although another study reported that prevalence of *K. inornata* increased with fish length (Arnott et al. 2017). In general, nothing is known about the routes of infection by *Kudoa* spp., and larger fish may be at greater risk of infection simply because more area is available for uptake of the putative infective stage, as suggested earlier for metazoan parasites (Poulin 2000). Alternatively, if the route of *K. thyr sites* infection is via the buccal cavity or gut, similar to that of *Henneguya ictaluri*, the cause of proliferative gill disease in channel catfish *Ictalurus punctatus* (Belem & Pote 2001), a higher rate of infection may occur in larger fish as a result of an elevated feeding rate. The increased occurrence of infection in larger fish may also be related to differential development of skeletal muscle among the IWGs. In both trials in the present study, the salmon were graded from common stocks, and on average, larger IWGs included individuals with higher growth rates. New, smaller muscle fibres are continuously recruited to the skeletal muscle during growth in salmon (Johnston et al. 2000) and a high proportion of these newly recruited fibres is expected to occur in rapidly growing fish (Weatherley et al. 1988). Thus, we predict a higher proportion of newly recruited muscle fibres in fish from the large IWGs; however, nothing is known about the affinity of these fibres for early *K. thyr sites* developmental stages (Young & Jones 2005, Marshall et al. 2016) or the subsequent proliferative development of the parasite.

It is important to differentiate our observations from the large body of knowledge that describes increasing prevalence or intensity of myxozoan infections with size (or age) in wild or cultured fish (Lom & Dyková 1992). The positive correlation of *Kudoa ciliatae* prevalence with body size was suggested to be related to the cumulative effects of prolonged exposure in juvenile Indo-Pacific whiting *Sil-*

lago maculata (Hallett et al. 1997). Similarly, accumulation or proliferation may explain the increased prevalence of *Ceratomyxa merlangi* and *Myxidium gadi* with larger size classes in whiting *Merlangius merlangus* from the Black Sea (Özer et al. 2017). The greater prevalence of *Sphaerospora dicentrarchi* in larger, older European seabass *Dicentrarchus labrax* was suggested to have been related either to a cumulative process, or to the greater consumption of or increased proximity to alternative hosts (Castro et al. 2018). Given the limits imposed on the interval of exposure in the present study, accumulation of infection does not explain our findings. Nevertheless, our study indicates the importance of considering host size or intrinsic growth rate, independent of age-associated accumulation, when attempting to interpret myxozoan infection parameters among age classes of wild or captive fish.

In conclusion and refuting our hypothesis (risk of infection with *K. thyr sites* is increased in smaller post-smolt Atlantic salmon), data from 2 controlled trials revealed a significant host size effect in which the prevalence and severity of *K. thyr sites* were greater in larger Atlantic salmon post-smolts. While our data did not reveal a mechanism for this effect, the generation of treatment groups of distinct size from age-matched salmon suggests a role of growth rate. This research suggests 2 hypotheses to be tested in future research: newly recruited, smaller skeletal muscle fibres are more prone to infection with *K. thyr sites*, and infection severity and host size are statistically interacting factors that affect swimming performance in salmon.

Acknowledgements. This research was funded by Fisheries and Oceans Canada's Aquaculture Collaborative Research and Development Program with support from Marine Harvest Canada and Cermaq Canada. Thanks to Steve Cho, Holly Hicklin and An Yuxin for assistance with fish husbandry and histological processing and microscopy.

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