Acquired resistance to *Kudoa thyrsites* in Atlantic salmon *Salmo salar* following recovery from a primary infection with the parasite

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**A B S T R A C T**

The influence of prior infection with *Kudoa thyrsites* or host size on the susceptibility of Atlantic salmon post-smolts to infection with the parasite was investigated. Exposure to infective *K. thyrsites* in raw seawater (RSW) was regulated by the use of ultraviolet irradiation (UVSW). Naïve smolts were exposed to RSW for either 38 days (440 degree-days, DD) or 82 days (950 DD) after which they were maintained in UVSW. Control fish were maintained on UVSW only. Microscopic examination at day 176 (1985 DD) revealed *K. thyrsites* infection in nearly 90% of exposed fish but not in controls. Prevalence and severity of the infection decreased in later samples. Following a second exposure of all fish at day 415 (4275 DD), prevalence and severity were elevated in the UVSW controls compared to previously exposed fish groups, suggesting the acquisition of protective immunity. In a second experiment, naïve smolts were exposed to RSW at weights of 101 g, 180 g, 210 g or 332 g and the prevalence and severity of *K. thyrsites* in the smallest fish group were higher than in any other group. However, infection parameters in the 101 g fish were not different from those in control fish (495 g) exposed in the first experiment. Concurrently, RSW was screened for *K. thyrsites* ribosomal DNA (rDNA) and the number of rDNA copies L−1 seawater did not agree well with prevalence or severity of infections. Possible reasons for this poor agreement are discussed. Increased resistance to *K. thyrsites* was evident in Atlantic salmon post-smolts that have recovered from the infection, suggesting strategies for the mitigation of soft-flesh in market-size salmon.

**Statement of Relevance:** We showed that Atlantic salmon which had recovered from infection with *Kudoa thyrsites* were resistant to reinfection with the parasite, suggesting an acquired immunity and the possibility of vaccination as a management strategy. Quantitative detection of parasite DNA in seawater provides a tool for predicting the risk of infection with this economically important parasite.

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1. **Introduction**

*Kudoa thyrsites* is a myxozoan parasite that infects a broad range of marine fishes and has a global distribution (Moran et al., 1999a; Whipps and Kent, 2006). In the northeast Pacific Ocean, the parasite is prevalent in hake (*Merluccius productus*) (King et al., 2012) and causes soft-flesh or myoliquefaction, which is commercially important in Atlantic salmon (*Salmo salar*) reared in netpens in British Columbia (BC), Canada (Moran and Kent, 1999a; Henning et al., 2013). In the salmon, the parasite produces spore-filled plasmodia within skeletal muscle cells (Kabata and Whitaker, 1981; Stehr and Whitaker, 1986) and infections are cryptic without the development of clinical signs. In Atlantic salmon, laboratory infections resolve between 26 and 52 weeks post-exposure (Moran et al., 1999c), however very little is known about the progression of infection in production salmon. Following harvest, parasite-derived proteases degrade the muscle affecting the texture and value of the fillet (Funk et al., 2008) in proportion to the severity of infection (St-Hilaire et al., 1997; Dawson-Coates et al., 2003). In 2013, claims and discards associated with *K. thyrsites* cost a segment of the industry in BC 16.9 million Norwegian Krone (Marine Harvest, 2014). Although infections are acquired by exposure of susceptible fish to infective seawater, neither the life cycle of *K. thyrsites* nor other aspects of its host-parasite relationship are well understood.

Management strategies for the avoidance or prevention of *K. thyrsites* infections in Atlantic salmon are limited by the absence of parasite-specific vaccines or approved treatments; although dietary nicarbazin was shown to be effective at reducing the severity of infections (Jones et al., 2012). Instead, exposure to infection is managed through site selection and overall fish health is managed by optimizing smolt quality (e.g., nutrition, vaccination against bacterial infections) and stocking density. In BC, salmon production sites are defined as high or low risk based on a history of the occurrence, severity and outcomes of *K. thyrsites* infections (Karreman et al., 2003). Anecdotal information suggests that severity of infections among production salmon at high-risk sites can be lessened by prior residence of smolts at low-risk sites following transfer to the ocean (P. McKenzie, personal communication). In this scenario, the transferred smolts will be larger upon first...
residence in high-risk sites therefore these observations do not distinguish between the possible effects of smolt size and prior exposure in contributing to the reduced risk of infection. The purpose of this study was twofold: first to explore the possibility that prior exposure to the parasite increases the resistance of salmon to a subsequent exposure and second, to determine the effect of smolt size on the susceptibility of Atlantic salmon to *K. thyrsites*.

2. Methods

2.1. Fish and seawater

Juvenile Atlantic salmon *S. salar* from a single commercial hatchery stock were used in both experiments and maintained in a research aquarium at the Pacific Biological Station, Nanaimo, British Columbia. The aquarium was provided with seawater pumped from approximately 2 m below the low tide datum in Departure Bay and sand-filtered to remove 95% of particles with a mean diameter greater than ~68 μm (raw seawater, RSW). RSW was ultraviolet irradiated (UVSW; mean 713 mJ cm

-2, range 370–713 mJ cm

-2). Its temperature ranged from 8.2 to 13.6 °C and mean salinity was 29.2 PSU (range 26.9–31.0 PSU) (Fig. 1). The fish were smolted onto UVSW in 6500 L flow-through tanks and acclimated for a minimum of 2 weeks prior to experimentation. Salmon were fed a commercial pelleted diet at a daily rate of 1% weight. Water temperatures.

2.2. Design

2.2.1. Experiment 1

Fish were allocated into 6 × 2500 L tanks (*n* = 150 tank

-1) having flow rates of 40 L UVSW min

-1 and two tanks were assigned to each of 3 treatment groups: UVSW only, RSW for 38 days (440 DD) and RSW for 82 days (950 DD). All fish were maintained in UVSW following the RSW exposure. Muscle samples were collected from 50 fish tank

-1 at day 176 (1985 DD) and day 346 (3500 DD) following the onset of exposure and from 13–20 fish tank

-1 at day 415 (4275 DD). Immediately following the last sampling, all remaining fish were exposed to RSW for 45 days (530 DD) then maintained in UVSW until day 596 (6225 DD) when final samples were collected (*n* = 25–30 tank

-1) (Table 1). For sampling, fish were sedated in 0.25 mg L

-1 Aquacalm (Syndel Laboratories, Canada) and killed in 250 mg L

-1 MS-222 (Syndel Laboratories). Three 1 cm

3 skeletal muscle samples dissected from each fish immediately after euthanasia were fixed in 10% neutral buffered formalin (NBF) for 24–48 h then transferred to 70% isopropanol.

2.2.2. Experiment 2 — effect of size

Fish were allocated into 12 × 1800 L tanks (*n* = 50 tank

-1) having flow rates of 30 L UVSW min

-1. Three tanks were assigned to each of 4 trials that were conducted sequentially over 17-months (Table 2). In each trial, fish were exposed to RSW followed by maintenance in UVSW. Exposure times varied with each trial (see Table 2). In each trial, fish weight in each trial was estimated from the combined wet weight of 20 fish. At the termination of each trial fish were weighed and muscle samples were collected and fixed as described above. RSW was screened for *K. thyrsites* rDNA as described in Section 2.4.

2.3. Histology

In both experiments, NBF-fixed samples from up to 20 randomly-chosen fish tank

-1 were processed for histological analysis and examined by light microscopy as described earlier (Jones et al., 2012). An infection severity index for each fish was calculated as the arithmetic average number of *K. thyrsites* plasmodia mm

-2 from the three muscle sections. The median infection severity for the treatment group was estimated from individual severity indices of infected fish.

2.4. Filtration of environmental DNA (eDNA) from seawater

During RSW exposures in Exp. 2, a standardized weekly regime was established (Monday, 1300 h–1400 h) in which 3 RSW samples ranging from 30 to 50 L were collected and immediately filtered through 90 mm, mixed-ester nitrocellulose membranes (5.0 μm pore size; Fisher Scientific, USA) which were then stored at −20 °C. Funnels and associated apparatus were rinsed with freshwater and air-dried between samples. The membrane was aseptically cut into quarters each placed into a separate microcentrifuge tube (in ~2 mm strips) and eDNA extracted using the DNeasy Plant Kit (Qiagen, USA) and stored at −20 °C.

2.5. Standard development

A 154 bp *K. thyrsites* small subunit (SSU) rDNA sequence (see GenBank accession number AP031412) was amplified using PCR primers Kt18SF-BC (5′-TGG TGG CCA AAT CTA GGT T-3′), a single nucleotide modification of the forward primer described in Funk et al. (2007),

### Table 1

Infections with *Kudoa thyrsites* in Atlantic salmon *Salmo salar* following primary and secondary exposures to infective seawater (experiment 1).

<table>
<thead>
<tr>
<th>Time&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Infection</th>
<th>Primary exposure&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Days Degree-days</td>
<td>Prevalence</td>
<td>Prevalence</td>
</tr>
<tr>
<td>176 1985</td>
<td>0</td>
<td>82.5</td>
</tr>
<tr>
<td>346 3500</td>
<td>5</td>
<td>60.0</td>
</tr>
<tr>
<td>415 4275</td>
<td>0</td>
<td>32.5</td>
</tr>
<tr>
<td>596 6225</td>
<td>92.5</td>
<td>35.0</td>
</tr>
</tbody>
</table>

<sup>a</sup> Since onset of primary exposure.  
<sup>b</sup> Duration of exposure to non-irradiated raw seawater in days (degree-days) (see text for methods).  
<sup>c</sup> Percent infected (*n* = 40).  
<sup>d</sup> Median plasmodia mm

-2 (25th, 95th percentiles; see text for methods).  
<sup>e</sup> Two infected samples: 0.281 and 3.082 plasmodia mm

-2.  
<sup>f</sup> n = 34.  
<sup>g</sup> 181 days after a 45 day secondary exposure.

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![Fig. 1. Temperature (dashed) and salinity (solid) of raw seawater from Departure Bay, British Columbia, Canada used in this study between June 2013 and July 2014.](image-url)
and SA-3 (5'-GGTTCCA TGC TAT AAC ATT CAA GC-3') (S. Byrne, personal communication). The probe was ligated into pCR2.1-TOPO and used to transform One Shot® TOP10F Escherichia coli for plasmid propagation (Invitrogen, USA). Selected colonies were grown in LB broth (Sigma, Canada) supplemented with 50 μg ml⁻¹ ampicillin (Sigma). Cultures were screened by PCR in 25 μl reactions containing 1× of Platinum Taq buffer (Invitrogen, USA), 0.2 μM dNTPs, 0.4 μM each forward and reverse primer, 0.5 U of Platinum Taq and 5 μl of template DNA. The reaction profile was 95 °C for 3 min followed by 40 cycles of 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. PCR products were visualized on 3% agarose gels stained with SYBR Safe (Invitrogen) and those with single-bands were purified (ExoSAP-IT, USB Corporation, USA) and sequenced in 10 μl reactions: 1 μl of purified product, 1× Big Dye Terminator mix (Applied Biosystems, USA), 1× Big Dye Terminator buffer (Applied Biosystems) and 0.5 μl of 3.2 pmol sequencing primers (described above). Following purification (Dye EX 2.0 Spin Kit, Qiagen), products were sequenced on an ABI 3130xl genetic analyser and sequences edited in Sequencher 5.1 (Gene Codes Corporation, USA).

Plasmid DNA was purified from PCR-positive bacteria using the PureLink Kit (Invitrogen), linearized in 16 μl water, 2 μl buffer (NEB2), 1 μl plasmid DNA and 1 μl HindIII restriction endonuclease, quantified (Nanodrop, Thermo Scientific, USA) and stored at −80 °C.

2.6. Quantitative PCR amplification of DNA from filter membranes

Reaction conditions and probe targeting K. thyrsites SSU rDNA were similar to Funk et al. (2007) and the primers were described above. Briefly, each 25 μl reaction included 0.3 μl forward primer, 0.45 μl reverse primer, 0.1 μl 6-carboxyfluorescein-labelled probe, 12.5 μl of Path ID reaction mix (Invitrogen), 6.65 μl of molecular grade water and 5 μl of template DNA. All samples and standards were run in triplicate on 96-well plates which included a serial dilution of 1 × 10⁸ to 1 × 10² copies μl⁻¹ to 1 × 10² copies μl⁻¹ of linearized standard DNA. Molecular grade water served as a negative template control. Specificity of the qPCR was assessed by amplifying DNA from purified myxospores (Henneguya salminicola, Chloromyxum sp., Myxobolus cerebralis) or infected tissues (K. thyrsites, Myxobolus arcticus, Parvicapsula minibicornis, Ceratonova shasta, Sphaerospora elwaiensis, Loma salmonae, Neoparamoeba perurans). Ct values were determined with threshold set to 0.1. For each standard reaction, efficiency, E was estimated by [(10⁻¹·5 − 1) × 100] and copy number, C by [(Ct − b) S⁻¹], where S is the slope and b the intercept.

2.7. Statistics

The statistical significance of differences in median fish weights at all times was tested by using the Kruskal–Wallis 1-way ANOVA on ranks (KW) followed by pairwise Dunn’s test for multiple comparisons. Similarly, the significance of differences in median infection severity among tanks and treatment groups was tested by KW tests followed by pairwise Mann–Whitney rank sum tests. Medians along with 25th (Q1) and 75th (Q3) percentiles are shown. Outlier Ct values (median > (Q3 + 1.5 × IQR)) were removed from further analysis and the remaining values standardized as copy number (C) L⁻¹ filtered seawater. The statistical significance of differences among median C⁻¹ values was tested by using the Kruskal–Wallis test. Pairwise comparisons of aggregate C⁻¹ values were tested using Mann–Whitney rank sum tests. Differences were considered statistically significant when p < 0.05.

3. Results

3.1. Effect of prior exposure

Fish were exposed to raw seawater (RSW) on days 0 and 415 and sampled on days 176, 346, 415 and 596. Between days 176 and 415, the overall prevalence of K. thyrsites in RSW-exposed fish declined from 88.8% to 36.5% (Table 1). Coincidentally, the overall median severity declined from 1.05 to 0.05 plasmodia mm⁻² (Table 1). The declines in median severity were statistically significant between days 176 and 346 (p = 0.002) and between days 346 and 415 (p < 0.001). No infections were detected in control (UVSW-exposed) fish at day 176 or 415, and 2 of 40 controls were infected at day 346 with severities of 0.28 and 3.08 plasmodia mm⁻². At no time were differences in median severity between replicate tanks and between the 38 day and 82 day primary exposure groups statistically significant (p > 0.225). By 181 days following the onset of the second exposure, infections were detected in 92.5% of the previous UVSW controls, in 35% of the 38 day-exposed and in 32.5% of the 82 day-exposed salmon (Table 1). Compared with controls, median severity was significantly lower in both previously exposed groups (p < 0.001). Median weights at each of the sampling times were 267.0 g (228.0, 301.5), 470.5 g (410.2, 514.1) and 807.0 g (747.0, 905.0), respectively.

3.2. Effect of fish size

Fish were exposed to RSW in 4 trials between June 5, 2013 and October 10, 2014 (Table 2). The difference in median end weights between any two trials, except trials 2 and 3, was statistically significant (p < 0.05) and differences in median weights among tank replicates in all trials were not statistically significant (0.051 < p < 0.575). Among trials, the prevalence of infection with K. thyrsites ranged from 67% to 87% (Table 2). The median severity was higher in the first trial compared with any other (p < 0.05); however, differences among severities in the remaining trials were not statistically significant (p > 0.05). In all trials, differences in median severity among tank replicates were not statistically significant (0.118 < p < 0.890).

<table>
<thead>
<tr>
<th>Trial</th>
<th>Dates</th>
<th>RSW</th>
<th>Weight (g)</th>
<th>Infection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Start</td>
<td>End</td>
<td>Start</td>
<td>Endb</td>
</tr>
<tr>
<td>1</td>
<td>Jun-5</td>
<td>Dec-12</td>
<td>45 (515)</td>
<td>101</td>
</tr>
<tr>
<td>2</td>
<td>Aug-27</td>
<td>Mar-24</td>
<td>45 (532)</td>
<td>180</td>
</tr>
<tr>
<td>3</td>
<td>Dec-10</td>
<td>Jul-24</td>
<td>56 (517)</td>
<td>210</td>
</tr>
<tr>
<td>4</td>
<td>Apr-10</td>
<td>Oct-10</td>
<td>53 (520)</td>
<td>332</td>
</tr>
</tbody>
</table>

See text for methods.

a Duration of exposure to non-irradiated raw seawater in days (degree-days).
b Median weight (25th, 75th percentiles).
c Percent infected (n = 60).
d Median plasmodia/mm² (25th, 95th percentiles).

Table 2: Infections with K. thyrsites among Atlantic salmon Salmo salar exposed to infective seawater in four experimental trials (experiment 2).
Atlantic salmon were exposed to infective seawater by periodic cessation of UV-irradiation of RSW and first examined for infection with *K. thyrsites* 176 days or 2000 degree-days (DD) later. In previous work, Atlantic salmon became infected with *K. thyrsites* following exposures to RSW from Departure Bay (Moran et al., 1999b; Jones et al., 2012) and Moran et al. (1999c) showed that the peak plasmodium density occurred at approximately 25 weeks or 2000 DD. The efficacy of UV irradiation in negating the infectivity of seawater was most evident from the absence of infection in any of 40 UVSW controls in comparison to the 89% prevalence among 80 salmon that had been exposed to RSW and sampled at 176 day. In addition, infections were not detected in 40 UVSW controls at 415 day. Previous research has shown that a UV dose of 44 mJ cm\(^{-2}\) prevented *Kudoa neurophila* infections in *Latris lineata* (Cobcroft and Battaglene, 2013). Similarly, maximum UV doses of 68 and 216 mJ cm\(^{-2}\) prevented *Kudoa yasunagai* and *Kudoa annamienensis* infections in *Seriola lalandi* and *Seriola quinqueradiata*, respectively (Shirakashi et al., 2014). In a static freshwater system, a UV dose of 1300 mJ cm\(^{-2}\) inactivated *M. cerebralis* triactinomyxons (TAMs) and prevented infections in juvenile rainbow trout at doses of 1400 and 14,000 TAMs fish\(^{-1}\) (Hedrick et al., 2000). In the present study, the rare detection of infection in UVSW controls sampled at 346 day may have been because of 10 system-level disruptions of UV-irradiation, each lasting from 10 min to 2 h. Despite this, our data indicate that a mean UV-irradiation of RSW at 350 mJ cm\(^{-2}\) was effective at significantly reducing the number of *K. thyrsites* plasmodia in Atlantic salmon.

The present data showed that in Atlantic salmon, complete or partial recovery from *K. thyrsites* infection is associated with an increased resistance to subsequent homologous challenge. The prevalence and severity of *K. thyrsites* infections in Atlantic salmon declined between days 176 and 415, regardless of the duration of primary exposure. Prevalence at the latter time was approximately 60% lower and median severity over 25-fold lower suggesting that in some fish, infections were resolved while in others they persisted at much reduced severity. These observations agree with earlier researchers who found that *K. thyrsites* infection in laboratory-exposed Atlantic salmon are diminished 1 year after initial exposure and that this recovery is associated with a chronic inflammation of the skeletal muscle (Moran and Kent, 1999; Moran et al., 1999c). Following re-exposure at day 415, the prevalence and severity of infections among controls were elevated, similar to those observed following the initial exposure of naïve fish, whereas on average, infections among the previously exposed fish did not change from pre-exposure levels. Recovery from infections with myxozoans or other parasites has been documented in salmonids and in the cases of *Cryptobia salmositica*, *Ceratomyxa* (= *Ceratona*) *shasta*, *L. salmonae*, *Gyrodactylus derjavini*, * Diplodostomum spathaceum* and *Tetracapsuloides bryosalmonae*, recovered fish are resistant to homologous challenge (Jones and Woo, 1987; Bartholomew, 1998; Speare et al., 1998; Kent et al., 1999; Lindenström and Buchmann, 2000; Karvonen et al., 2005; Schmidt-Posthaus et al., 2012). Similarly, infections with low-virulence strains of *C. salmositica* or *L. salmonae* increase host resistance against subsequent virulent homologous challenge, indicating vaccine potential (Woo and Li, 1990; Sanchez et al., 2001). In the case of *K. thyrsites*, phagocytosis of myxospores and other parasite debris from ruptured plasmodia by macrophages during the recovery phase of infection (Moran et al., 1999c) indicates immunological surveillance by the Atlantic salmon host. Similarly, increased severity of *K. thyrsites* during smolitification or sexual maturation in Atlantic salmon is inferred to be associated with depressed host immunity (Moran and Kent, 1999; St-Hilaire et al., 1998). To date however, there are no data on mechanisms of the acquired protective immunity to *K. thyrsites* in recovered Atlantic salmon. Generally, defence mechanisms associated with the increased resistance to parasite challenge in recovered salmonids include transcription of innate and adaptive immune genes and
production of non-specific and specific humoral and cellular factors (Alvarez-Pellittero, 2008).

In assessing the effect of fish size on risk of infection, our results showed that fish exposed to RSW at 101 g had a significantly higher median severity of infection in comparison to heavier fish exposed at weights up to 332 g. Contradicting this finding however, there was no statistically significant difference between the infection severity in the fish exposed at 101 g and controls in the first experiment exposed at 495 g (analysis not shown), suggesting size alone is not a useful predictor of risk. Alternatively, we considered whether variable infection levels among the trials may have been related to seasonal differences in the infectivity of Departure Bay RSW, as suggested earlier by Moran and Kent (1999). In that study, the parasite was detected in Atlantic salmon exposed in April, June, August or October but not in those exposed in December, January or March. Warmer water temperatures in Departure Bay in 2013–14 compared with 1995–96 (see Fig. 2 of Moran and Kent, 1999) may have been permissive to winter transmission, possibly explaining why we were able to detect infections throughout the year. The abundance of K. thyrsites rDNA in RSW was measured using a quantitative PCR (qPCR) similar to that described earlier (Funk et al., 2007). Evidence of significant variation among trials was observed, with the highest abundance of parasite rDNA occurring during trial 2. Coincidentally, the prevalence and severity of K. thyrsites infections were relatively low indicating that our RSW screening method was not a useful predictor of risk. Despite this, seasonal analysis showed the abundance of parasite rDNA in Departure Bay was greatest among samples collected in summer and least in winter (not shown), in agreement with the previously observed pattern of RSW infectivity. Poor agreement between the qPCR data and infectivity of RSW was likely related to inadequacies in our methods. For example, we screened at most 0.05% of the approximately 2.12 × 10^6 L RSW flowing through each tank during intervals when UV-irradiation was discontinued, rendering the samples unlikely to be fully representative of the actual exposure. Furthermore, the qPCR is unable to distinguish between infective and non-infective parasite stages and likely therefore, to overestimate the risk of infection. The identity of the K. thyrsites infective stage is not known and there is no information on relative abundances of putative actinospores and myxospores, the latter of which are not infective to naïve Atlantic salmon (Moran et al., 1999b) but released into seawater during the decay of naturally-infected hosts. Until we have a better understanding of actinospore structure and the ratio of actinospores to myxospores in RSW, there is little value in estimating actinospore concentrations from qPCR data. Finally, although we demonstrated that the qPCR was reasonably specific, our samples may have included rDNA from other enzootic Kudos spp. (i.e., K. paniformis) since a similar test amplified K. miniauriculata rDNA (Funk et al., 2007). We will assess in ongoing work the extent to which this rDNA abundance, obtained from a more frequent RSW sampling regime than used here, correlates with infection levels in concurrently exposed salmon. In contrast to K. thyrsites in Departure Bay, the highest levels of K. yasunagai rDNA in Tanabe Bay, Japan occurred mid-winter, coincident with the coolest water temperatures (Ishimaru et al., 2014). Alternatively, peak concentrations of rDNA from Ceratomyxa puntazzi in the Mediterranean Sea, occurred in late summer to early autumn, somewhat later than the warmest water temperatures (Alama-Bermejo et al., 2013). Similar approaches have been applied for the detection of DNA belonging to myxozoan fish pathogens in freshwater (Hallett and Bartholomew, 2006; Griffin et al., 2009).

In conclusion, the significant reduction in the prevalence and severity of K. thyrsites following re-exposure of previously infected salmon compared with controls, suggests opportunities for novel management strategies for soft-fleshed cultivars of Atlantic salmon. Such strategies may include the controlled early exposure of salmon to K. thyrsites as a form of vaccination. Further research is required to optimize this vaccine effect by studying the timing and duration of the primary exposure. In addition and with some refinement, the water-screening method described here will assist in the definition of risk of infection among production sites.

Acknowledgements

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