Long term epidemiological survey of *Kudoa thyrsites* (Myxozoa) in Atlantic salmon (*Salmo salar* L.) from commercial aquaculture farms.

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Running title: Epidemiology of *K. thyrsites* in farmed Atlantic salmon.

Abstract

Kudoa thyrsites is a myxozoan parasite, which encysts within myocytes of a variety of fishes. While infected fish appear unharmed, parasite derived enzymes degrade the flesh post mortem. In regions of British Columbia (Canada) up to 4-7 % of fillets can be affected, thus having economic consequences and impacting the competitiveness of BC's farms. Infections were monitored in two farms having high (HP) or low (LP) historical prevalence. Prevalence was determined by PCR of muscle, and intensity was measured by histology. In parallel, fillet tests were used to quantify myoliquefaction. At each farm, 30 fish were sampled monthly during the first year followed by nine samplings during year two. Infections were detected by PCR after 355 and 509 degree days at LP and HP farms, respectively. Prevalence reached 100 % at the HP farm by 2,265 degree days and then declined during the second year, whereas it plateaued near 50 % at the LP farm. Infection intensities decreased after one year at both farms. Blood was PCR positive at both farms between 778-1,113 degree days and then again after 2,000 degree days. This is the first monitoring project in a production setting and compares data between farms with different prevalence.

Keywords

Immunity, Intensity, Histopathology, Myoliquefaction, Prevalence, Pseudocysts

Introduction

The genus *Kudoa* (Myxozoa) is composed of over 90 species parasitic on a wide range of marine and estuarine teleosts, and one species from the North-Pacific giant octopus *Paroctopus dofleini* (Wülker) (Yokoyama & Masuda 2001). Most *Kudoa* species are histozoic, infecting a variety of tissues including muscle, kidney, ovary, and brain (Moran, Whitaker & Kent 1999c). Infections of the skeletal muscle are the most common and are characterized by visible cysts or microscopic intracellular pseudocysts (Moran *et al.* 1999c). Although there are no reported effects on the physiology or life-span of the hosts from skeletal muscle infections, their impact on commercial fisheries and aquaculture is due to the presence of visually unappealing cysts or post-mortem softening of the flesh (Moran *et al.* 1999c). *Kudoa thyrsites* (Gilchrist 1924) causes post-mortem myoliquefaction, otherwise known as soft-flesh syndrome, in 37 fish species including Pacific hake, *Merluccius productus* (Ayres), Mahi mahi, *Coryphaena hippurus* (L), Atlantic mackerel *Scomber scombrus* (L), coho salmon, *Oncorhynchus kisutch* (Walbaum), and Atlantic salmon, *Salmo salar* (L) (Whipps & Kent 2006; Moran *et al.* 1999c; Langdon 1991; Kabata & Whitaker 1981; Harrell & Scott 1985; Kabata, Whitaker & Bagshaw 1986; Levsen, Jørgensen & Mo 2008). *K. thyrsites* has a widespread distribution with endemic strains in most temperate regions, including the North-East Pacific, North-West Europe, Japan, Southern Australia, Chile, and South Africa (Whipps & Kent 2006), and is the most frequently studied species of the genus. Economic consequences of soft-flesh syndrome are high in aquaculture facilities located in regions having high prevalence of infection. Although infection prevalence can be monitored within affected farms by PCR or examining fillets for manifestation of soft-flesh, there are no external clinical signs to identify and cull infected fish. Therefore, the effects are not realized until after processing and accrual of all production costs. In British Columbia, Canada annual costs to the Atlantic salmon aquaculture industry due to *K. thyrsites* infections reach millions of dollars, e.g. in 2002 losses were estimated at 50 million CAD (Funk, Raap, Sojonky, Jones, Robinson, Falkenberg & Miller 2007).

Myoliquefaction is caused by enzymatic degradation of the host musculature. In *M. productus*, myofibrils in close proximity to the pseudocysts of *K. thyrsites* and *K. paniformis* infections are affected while the fish is alive (Stehr & Whitaker 1986). Most enzymes responsible for liquefaction are thought to be adsorbed or metabolized by the living host, but accumulate after the host dies (Willis 1949). Funk, Olafson, Raap, Smith, Aitken, Haddow,

Wang, Dawson-Coates, Burke & Miller (2008) identified a cysteine protease, Cathepsin L, as the responsible enzyme. Both Funk et al. (2008) and Martone, Spivak, Busconi, Folco & Sánchez (1999) demonstrated that the enzyme is associated with pseudocysts of *Kudoa*. Earlier, Patashnik, Groninger, Barnett, Kudo & Koury (1982) concluded that while the proteolytic enzymes were associated with pseudocysts, they were not produced by the mature myxospores. In Atlantic salmon, the extent of post mortem myoliquefaction is correlated with parasite intensity measured as the number of pseudocysts/mm^2 (Funk et al. 2007), the number of myxospores (Dawson-Coates, Chase, Funk, Booy, Haines, Falkenberg, Whitaker, Olafson & Pearson 2003; St-Hilaire, Hill, Kent, Whitaker & Ribble 1997), or the quantity of nucleic acids of *K. thyrsites* origin (Funk et al. 2007). Similar correlations have been documented in infected *M. productus* (Zhou & Li-Chan 2009; Samaranayaka, Ho & Li-Chan 2007).

Myxozoa have an alternating two host-life cycle where development in each host concludes with the release of one of two types of infective spores. Myxospores are typically produced during maturation within a vertebrate host (usually fish and amphibians) and actinospores are formed within an invertebrate host following gamogamy (Kent, Andree, Barhtolomew, El-Matbouli, Desser, Devlin, Feist, Hedrick, Hoffman, Khattra, Hallett, Lester, Longshaw, Palenzeula, Siddall & Xiao 2001). Although *K. thyrsites* has many piscine hosts, fish to fish transmission via intubation of infected tissue has not been successful (Moran, Whitaker & Kent 1999b), such as demonstrated for *Enteromyxum* species (Sitjà-Bobadilla & Palenzuela 2012). Instead, infection occurs after exposure to seawater (Moran *et al.* 1999b) and therefore the life-cycle of *Kudoa* is presumed to be similar to other two-host myxozoa. The identity of the invertebrate host(s) remains unknown and all the stages thus far have been described in fish. *K. thyrsites* is first found as small multicellular plasmodia located intracellularly within myocytes, and later develops into larger pseudocysts containing disporoblasts, developing and mature myxospores, and undifferentiated cells (Willis 1949). Typically mature myxospores are located in the interior of the pseudocysts and undifferentiated cells along the periphery (Morado & Sparks 1986; Moran, Margolis, Webster & Kent 1999a).Without the ability to identify and culture the infective stage from the putative invertebrate host, research has largely been focused on monitoring and diagnostic development.

Thus far, all population level surveys have been conducted at the Pacific Biological Station's experimental net pen and tank facility in Nanaimo (British Columbia), a research

facility located in the Strait of Georgia approximately 200 km Southeast of most affected aquaculture facilities (Moran & Kent 1999; Moran *et al.* 1999a; Moran *et al.* 1999b). Most data were collected during the first year of exposure using light microscopy except for (Moran *et al.* 1999a), where PCR was also used. These previous studies showed that muscle tissue from Atlantic salmon became infected within the first few months of exposure and that infection prevalence declined after 9-12 months (Moran & Kent 1999; Moran *et al.* 1999a). Inflammation occurred in response to lysis and degeneration of infected muscle fibres throughout most of the infection period, but eventually resolved (Moran *et al.* 1999a). No earlier stages have been described, but Moran *et al.* (1999a) detected the parasite in multiple tissues, including blood, by PCR, especially during the initial period of infection. Blood was suspected to be the route of dispersion within fish following successful transmission via intraperitoneal injection of blood from a chronically infected fish (Moran *et al.* 1999b).

Industry monitoring programs have shown that there is regional variation in the level of infection and consumer claims within British Columbia's waterways (Morrison & MacWilliam 2010b). Fish raised in farms on the West Coast and in the northern most reaches of Vancouver Island have lower effects of *K. thyrsites* than those in farms in the Discovery Island regions off the central inside coast of Vancouver Island (Morrison & MacWilliam 2010b; Karreman, Saksida, Jones & Stephen 2003). It is assumed that fish farmed in low prevalence regions have less exposure to the parasite, presumably due to a differential distribution of the alternate host. However other factors affecting host parasite interactions, such as water temperature are also known to affect the outcome of myxozoan infections of fish (Schmidt-Posthaus & Wahli 2015).

The primary objectives of the current study were to combine PCR detection, histology, and fillet testing to compare the progression of infection throughout the two year marine grow-out phase at two commercial sites located in regions having different predicted infection outcomes. Our data, based on multiple samples per fish, provide the most detailed description of the progression of infection, especially during the second year of exposure. In addition, this is the first data obtained from a less affected population.

Materials and Methods

Fish farms and sampling procedures

Two commercial Atlantic salmon farms were sampled throughout their grow-out production cycle. Farms were chosen based on the historical records of fillet discard rates due to *K. thyrsites,* and classified as either being high (4.0 – 7.6 %) or low (0.5 – 3.4 %) (Morrison & MacWilliam 2010b). The predicted high prevalence (HP) farm was located in the Discovery Islands between central Vancouver Island and the mainland of British Columbia, Canada. The predicted low prevalence (LP) farm was located in the Queen Charlotte Strait, northeast of Vancouver Island. Fish were entered on April 3rd and May 17th 2010 to each site respectively (Tables 1 and 2). Fish at the LP site were moved to an adjacent farm in January 2011. At each farm, 30 fish were sampled (blood and muscle) monthly for 15-17 months and then at 2.5 month intervals for the final three samplings totalling more than 500 fish (Tables 1 and 2). Sampling ended shortly before commercial harvest at approximately 24 months after seawater entry. Sampled fish were randomly chosen from the same population (net pen) using a box seine and a dip net. Fish were reared using the standard commercial procedures of the farms. Water quality parameters, including temperature, salinity and dissolved oxygen were recorded daily throughout the sampling time following established farm procedures.

At each sampling time, fish were quickly captured and placed in a bucket containing a lethal dose (300-500 mg/L) of tricaine methanesulfonate (Syndel Laboratories Ltd., Canada) or by irreversible percussive stunning. Then, blood was collected within 2 min after anaesthesia from the caudal vein using EDTA coated vacutainers (BD Vacutainer®) and placed on ice until centrifugation. Blood was centrifuged at 3,000 *g* for 10 min at 4 °C, and plasma and blood cells were frozen at – 80 °C. Fish were held on ice until further sampling. Muscle samples were collected from eight tissue sites (Fig. 1) within 1-4 h after capture, or within the next 12-18 h from fish transported on ice and stored at 4ºC. Muscle samples were frozen on dry ice and transferred to a -80 °C freezer for PCR analysis or fixed in 10% neutral buffered formalin for histology. Wet weight, fork length and sex were recorded for each fish.

Fillet assessment

All fish weighing 1 kg or greater were used for fillet assessment to measure the effects of *K. thyrsites* (pit counts and manifestation of myoliquefaction) (Tables 1 and 2). As growth was

slower at the LP farm, fewer fish were analyzed (n=181) than at the HP farm (n=350) (Tables 1-2). Both left and right fillets were collected from these fish either during sampling, or within 12-18 hours from whole fish transported on ice and stored at 4 ºC. Fillets were stored flat, skin side together in sealed bags (3-4 ºC). Each fillet was examined for pits at one, three, and seven days after sampling. Pits measuring less than 2 mm diameter, or those due to gaping or mechanical damage were not counted. Fillet classification followed a modified version of the grading system described by Dawson-Coates *et al.* (2003). Each fish was assigned a grade (K0 to K6) depending on the final count of pits from both fillets on day seven. K0 to K3 grades had counts of 0, 1-10, 11-20, and 21-40, respectively. K4 fillets ranged from 41-120 isolated and well defined pits with small patches of myoliquefaction. A K5 fillet either had greater than 120 pits or indefinable pits due to widespread myoliquefaction. Fillets that were extensively liquefied to the point where the flesh was able to slide off the skin were categorized as K6.

PCR analysis

DNA was extracted from 10 µl of red blood cells diluted in 190 µl phosphate buffered saline or from 25-30 mg of muscle tissue, using a QIAGEN DNeasy® Blood and Tissue kit according to manufacturer's instructions. Nested PCR, targeting the ribosomal small subunit (*rDNA-SSU*) of *K. thyrsites*, was performed in two stages using two primer sets (Table 3). All reactions were amplified for 50 cycles using a Mastercycler (Eppendorf) and Illustra^{TM} PuRe Taq^{TM} Ready-To-Go^{TM} PCR beads at an annealing temperature of 50 ºC. The resulting 178 bp amplicon was visualized on a 2% agarose gel. Between 3-5 PCR products from each tissue type were confirmed as *K. thyrsites* by sequencing by Macrogen (Seoul, Korea).

K. thyrsites is known to have patchy distribution throughout skeletal muscle (Funk et al. 2007), therefore false negatives can be expected when using muscle as an indicator of infection. To compensate, each fish was systematically tested before classifying it as positively or negatively infected. For example, if a fish was positive in tissue site A, it was called positive and no more PCR testing was done; however, if a fish was PCR negative at tissue site A, then site B was also tested and so on, up to four tissue sites per fish. In HP fish, all eight sites were analysed in order to evaluate the sensitivity of the test throughout the sampling period, but prevalence was calculated from the four samples collected on the left side of the fish as per the LP farm.

Histopathology

Up to ten PCR positive fish (based on the result from the first muscle site) were randomly chosen for the evaluation of the intensity of infection using histological examination of the muscle. See details of sampling numbers and dates in Tables 1 and 2. The total number of fish examined from the LP farm (109) was lower than from the HP farm (147) because less than ten fish were PCR positive in several samplings. All fish with PCR positive blood and an additional subset of fish with either light or heavily infected fillets were also included (n=43).

Myomere cross sections from the eight muscle sites were processed, embedded in paraffin, sectioned, and stained with Hematoxylin and Eosin (H&E) by the British Columbia Ministry of Agriculture and Lands in Abbotsford, British Columbia following standard protocols. The entire area of all sections was examined using an Olympus CX41 compound microscope at 200X magnification. The intensity of infection was calculated using total pseudocyst counts standardized to area. The area of the sections was determined by tracing photographs of each section using Photoshop v.6.0 and converting the traced areas into pixels using Image J v.1.44. Data was analyzed with and without counts from poor performers, defined as fish that weighed less than 40% of the monthly mean.

A subsample of 20 infected and one uninfected fish from the HP site were embedded in Technovit resin (Heraeus, Germany), sectioned at 2-3 μm, and Giemsa stained. Most selected fish were collected between 778 and 1,113 degree days and included fish that were PCR positive in blood and/or muscle, but otherwise negative through H&E screening. An additional eight fish with exceptionally heavy infections from 1,400, 1,675, and 2,013 degree days were also included. Sections from each fish were examined for stages prior to penetration of myocytes, developmental stages within myocytes, and cellular immune response.

Statistical analysis

All statistical analyses were performed using Statistics v.9. All data was validated for normal distribution and homogeneity of variance using frequency histograms and descriptive statistics otherwise non-parametric tests were used.

Results

The overall number of degree days and fish size were greater at the warmer, more southern HP farm. The average measured temperature during the study was 8.70 ºC at the HP farm and 8.44 ºC at the LP farm. Salinity and monthly dissolved oxygen levels were consistently lower at the HP farm (Tables 1-2).

Fillet analysis

Fillets from the HP farm consistently showed more pitting (after seven days of observation) than those from the LP farm throughout the entire period of analysis (Fig. 2; Table S1). An average of 34 % of fillets from the LP farm had one or more pits compared to 72 % at the HP farm. Very few fillets (4 %) from the LP farm were categorized as K4 – K6 compared to 27 % at the HP farm (Fig. 2, Table S1). There was no change in fillet manifestation within farms with increased production time or growth. There was no difference in manifestation in fish filleted at the farm compared to those filleted the following day (not shown). Fillet manifestation was sometimes visible the day following sampling (Day 1) but became more visible after four to seven days (Fig. S1). Analysis of results from the final six sampling points from HP fish showed a significant decrease in the percent of unaffected (K0) fillets ($P = 0.002$) and a corresponding increase in K3-K6 fillets ($p = 0.0054$) between days one and four (analysis of variance (ANOVA) followed by Tukey`s pairwise comparison). Changes between four and seven days were not statistically significant by pairwise comparison.

PCR sensitivity and sampling effort

K. thyrsites has an over dispersed distribution and multiple muscle samples per fish were necessary to compensate for false negative results (type two error) from PCR analysis. Assuming a negligible type two error rate after testing up to eight muscle samples, the percent of false negatives per number of tissue samples analysed from the HP farm was calculated. The proportion of fish incorrectly identified as uninfected had an inverse log relationship ($R^2 = 0.998$) to the number of tissue sites used (Fig. 3). Therefore, the false negative rate was approximately 33 % using one tissue site compared to 9 % when using four sites (Fig. 3). All results described hereon are based on four tissue sites per fish.

Monthly sampling effort at the HP farm, defined as the total number of PCR sites tested each month (maximum 120) was lowest (52 or less PCR reactions) between 2,265 and 4,017 degree days corresponding to a 90 % or greater prevalence (Fig. 4, Table 1). Sampling effort at the LP farm was highest between 622 and 1,518 degree days, and then varied between 62 and 92 PCR reactions for the duration of the sampling period (Table 2).

K. thyrsites prevalence of infection by PCR

K. thyrsites was first detected in muscle tissue earlier at the LP farm (38 days post entry = 355 degree days) than at the HP farm (58 days post entry = 509 degree days), though with the same prevalence of infection (13.3 %). Subsequently, infection prevalence increased at both farms until approximately 2,000 degree days, reaching higher values (90-100 %) in HP fish (between 2,265 and 4,017 degree days) than in LP fish (37- 73 %) (between 1,804 and 3,053 degree days). In both farms, there was a subsequent decline in the prevalence, though more pronounced in the HP farm. In any case, prevalence of infection was always higher in the HP than in the LP farm (Fig. 4).

K. thyrsites was first detected in the blood from HP fish at 778 degree days with a prevalence of 23.3 %. In subsequent samplings, prevalence ranged between 0 and 17 % (Table 4, Fig. S2). A similar pattern, although with later first appearance (975 degree days) and lower prevalence, occurred at the LP farm (Table 5). All fish, except one, having PCR positive blood were also PCR positive in the first muscle tissue site tested. This exception was PCR positive in the second tested muscle site.

Histologic diagnosis and histopathology

Pseudocysts were visible by histology at 1,113 and 975 degree days at the HP and LP farms, respectively. At the HP farm, pseudocyst detection matched between 80 to 100 % of muscle PCR positive fish from 1,400 to 3,300 degree days. However, later on these values decreased to 50 to 80 % (Table 4). At the LP farm, corresponding prevalence by histology was 100 % between 1,236 to 1,962 degree days, but later decreased to values between 33 and 67 % (Table 5). No pseudocysts were visible in the eight fish that were PCR positive in the blood at 778 and 1,113 degree days at the HP farm (Table 4), but they were visible in one of the two fish that were positive in blood at 975 degree days at the LP farm (Table 5). However, in subsequent

HP samplings (2,265 degree days onward) 92 % of the blood positive fish also had visible pseudocysts; most also exhibiting inflammation and heavy infections (Table 4). Coincidence of blood positives and pseudocysts was also high (80 %) in the LP farm between 2,682 and 4,068 degree days (Table 5).

Infection intensities, calculated from the cross sections from randomly selected PCR positive fish, were higher at the HP farm than the LP farm at similar degree days (Fig. 5). Intensity increased as *K. thyrsites* became visibly established in the muscle of PCR positive fish, reaching averages of 0.22-0.57 pseudocysts per mm^2 at the HP farm between 1,400 and 2,013 degree days. In contrast, average highs at the LP farm ranged between 0.12-0.13 pseudocysts per mm^2 between 1,518-1,962 degree days (Fig. 5). Intensity dropped in the subsequent samplings at both farms. Individual variation within each sampling time was high, such that statistically significant differences ($P < 0.05$) were only detected between the peak levels and those obtained at 5,392 degree days at the HP farm, and between peaks levels and those obtained at 4,068 and 5,694 degree days at the LP farm (identified by pairwise comparisons following a Kruskal-Wallis non-parametric ANOVA). Exclusion of poor performers did not affect the analysis, but these fish generally had higher than average parasite intensity. Intensities of infection obtained from different tissue sites of the fish (Fig. 1) were not significantly different (data not shown).

When the intensity of infection was analysed from 81 fish sampled between 2,901 and 6,389 degree days at the HP site, selected through having either high (K4-K6) or low (K0-K2) fillet manifestation levels a strong and significant positive correlation was found between intensity and fillet severity ($P< 0.05$) Spearman's rank correlation (not shown). However, the average number of pseudocysts in K4-K6 fillets was negatively correlated with increasing number of degree days at the HP farm (Fig. S3). Intensity of infection of K4-K6 fillets at 2,901 degree days was significantly different ($P < 0.05$) from that at two of the final three samplings (Kruskal-Wallis ANOVA), but there were no significant changes in K0-K2 during the same time period (Fig. S3).

Initial stages of the parasite occurred earlier at the LP farm (between 975 and 1,236 degree days) than at the HP farm (between 1,113 and 1,400 degree days). During this period many fish did not have any visible myxospores (Figs. 6A-B). Undifferentiated initial stages and disporoblasts were located along the pseudocyst periphery and mature spores filled the interior (Figs. 6C-D, 7A). Some pseudocysts appeared to have microvilli-like extensions on their

periphery (Fig. 6C). Most infected myocytes had a single pseudocyst situated near the center of the cell, but it was not uncommon for pseudocysts to be near the edge of the myocyte. Multiple infections were frequently observed, with a maximum of four seemingly distinct pseudocysts within a single myocyte. Branching of pseudocysts was not observed in any longitudinal section. Infected myocytes appeared to be clumped, rather than evenly dispersed, but this observation was not quantified. Putative early stages were observed in blood vessels (Figs. 6E-F), migrating through skin (Fig. 6G), or as multinucleate cells interstitially between myocytes (Fig. 6H). These unusual cells were rare and we were not able to conclusively identify them as *K. thyrsites* by conventional histology. Pseudocysts from older fish were filled with mature myxospores in a matrix of eosinophilic fibrous material (Fig. 7B). H&E stained polar capsules on developing spores were pink (Fig. 6D), but became refractile and pale-mauve with deeply stained pycnotic capsulogenic nuclei once mature (Figs. 6C, 7B).

Cellular immune response in the form of melanomacrophage centres appeared first at 1,113 and 1,518 degree days at the HP and LP sites, respectively (Tables 4 and 5). Many of these fish also had loose or phagocytosed myxospores within these foci (Tables 4 and 5, Figs. 7C-D). This evidence of inflammation was present throughout the entire sampling period, but decreased after 3,675 and 3,053 degree days at the HP and LP farms respectively (Tables 4 and 5).

By 1,400 degree days many pseudocysts appeared to have lost their integrity and myxospores, disporoblasts, and other undifferentiated cells were visible within and outside of myocytes (Fig. 7E). Disintegration of the surrounding myofibrils (Fig. 7E) was also notable on approximately half of these fish. The majority of fish (approximately 80%) having this attribute were dissected one day post mortem, so most of these effects were probably attributable to myoliquefaction rather than autolysis. Four fish from the high prevalence site, sampled within 30 min to four h of capture, had immune cells within the affected myocytes. On rare occasions, macrophages were present within muscle cells that had both degenerated pseudocysts and myofibril breakdown. However, in general, the most common form of immune response was inflammation presumably in response to completely degenerated myocytes rather than to intact myocytes.

Discussion

In the present survey, the progression of *K. thyrsites* infections at two operating commercial sites having different historical levels of soft-flesh manifestation at harvest is described. Pseudocyst development, host immune response and infection prevalence from the high prevalence site showed similarities to previous work at the Pacific Biological research station, located further South in the Straight of Georgia (Moran *et al.* 1999a; Moran & Kent 1999), but herein more sample points and both PCR analysis of multiple tissues and pseudocyst counts were used. All measures of infection were lower at the historically LP farm. Fillet manifestation results did not show any indication of change over time, but at the HP farm PCR prevalence and intensity of infection decreased near the end of the production cycle. At the LP farm, intensity was the only parameter that clearly decreased. PCR was the most sensitive detection method, detecting higher numbers of infected fish at earlier sampling times, but, also had false negatives in inverse proportion to the numbers of muscle tissue sites tested.

Fillet analysis

Pits counts were consistently lower at the LP farm throughout the sampling period , thereby confirming the original region based expectation. The values obtained here were higher than those of industry surveillance records, most likely due to warming during the 10-20 minute period of sample dissection compared to the rigorous temperature management of a commercial harvest. A positive correlation between temperature and myoliquefaction has been reported previously in Pacific whiting, *M. productus*, infected with *K. paniformis* (Patashnik et al. 1982). Although we could distinguish a clear difference in manifestation level between farms, we did not detect any change over time. Variation in the surface area of fillets from fish ranging from one to up to 14.5 kg may have impacted the results, but there was no apparent reduction in unequivocally severe fillets during the second year. Less subjective measures of myoliquefaction, such as the use of texture analyzers, show good correlation to pit counting (Dawson-Coates et al. 2003). Although PCR prevalence and pseudocyst density decreased with time, other changes in pseudocyst development, may have been contributing. For example, changes in pseudocyst morphology or size, or enzyme production over time were not measured.

However, pseudocyst density decreased during the final year within the subset of fish having high levels of myoliquefaction, was detected in highly which suggests that other factors such as pseudocyst development or size may have affect the degree of myoliquefaction.

Histological analysis

Intracellular pseudocysts were easily observed in various stages of development but detection and identification of earlier forms of infection and development proved challenging. Examination of muscle and blood vessels of younger and more heavily infected fish was inconclusive with the detection of a few seemingly foreign cells that could not be positively identified as *Kudoa*. Morado and Sparks (1986) described a 5-10 µm putative intracellular infective stage within host phagocytes in both juvenile and mature *M. productus*. These cells were described as typically unicellular but occasionally multicellular, and were described from fish with a range of infection levels including fish having light infections prior to spore development (Morado & Sparks 1986). Stages representing early infection may be difficult to observe if they are present in low numbers due to a slow accumulation of infection(s) or if they transition quickly to the intracellular stage. Identification of early infective and migratory stages of other myxozoa, such as, *Ceratomyxa shasta* (Bjork & Bartholomew 2009), *Henneguya ictaluri* (Belem & Pote 2001), and *Sphaerospora truttae* (Holzer, Sommerville & Wootten 2003), have benefited from challenges with high doses of infective actinospores and the application of in-situ-hybridization techniques.

Once within myocytes, mature myxospores tended to concentrate within the interior of developing pseudocysts and were surrounded by undifferentiated or sporogonic cells, as described from most hosts infected with *K. thyrsites* (e.g. Morado & Sparks 1986; Moran *et al.* 1999a). The thread-like extensions occasionally observed between the pseudocyst and host cell cytoplasm can be interpreted as membrane foldings of the primary cell. Using electron microscopy, Stehr and Whitaker (1986) described microvilli in *K. thyrsites* from *M. productus,* having dimensions of 0.5 µm in width by 0.9 µm in length. Older fish appeared to have larger pseudocysts, lacking undifferentiated cells and having an eosinophillic fibrous matrix that we did not observe in pseudocysts from younger fish. It is possible that these differences may provide an indication about when the fish acquired the infection, but detailed measurements were not within the scope of this survey. The size of the pseudocysts may also be a function of increased

myomere size in larger fish. Counts per mm^2 from different regions of the fish were not different, contrary to observations of mixed *Kudoa* infections in *M. productus* (Kabata & Whitaker 1985) and of *K. thyrsites* in *C. hippurus* (Langdon 1991).

Visible evidence of cellular immune response to *K. thyrsites* appears to be host dependent. The consistent inflammatory response with melanomacrophage centres and evidence of phagocytosis observed here was previously reported by Moran and co-authors (1999a), but Harrell and Scott (1985) reported an absence of inflammation in infected Atlantic Salmon. Inflammation, caused by *K. thyrsites*, has been observed in *C. hippurus* and *M. productus* (Langdon, Thorne & Fletcher 1992; Morado & Sparks 1986). No inflammation was observed in clupeoids, tubesnout *Aulorhynchus flavidus* (Gill), or mackerel *S. scombrus* (Langdon *et al.* 1992; Shaw, Herviot, Devlin & Adamson 1997; Levsen *et al.* 2008). Other members of the genus cause inflammation in Bocaccio *Sebastes paucispinis* (Ayres), White perch *Morone Americana* (Gmelin), and Pacific Hake *M. productus* (Whitaker, Kent & Sakanari 1996; Bunton & Poynton 1991; Morado & Sparks 1986). We did not see any evidence of encapsulation with collagen or fibroblasts, nor any form of melanisation, such as has been reported from infections of *K. thyrsites*, *K. paniformis* or *Kudoa rosenbuschi* in hakes *M. productus* and *Merluccius hubbsi* (Marini) (Morado & Sparks 1986; Martone *et al.* 1999; Stehr & Whitaker 1986; Kabata & Whitaker 1981). All of these reactions are characteristic responses to myxozoan infections of fish (Sitjá-Bobadilla 2008).

Loose or uncontained spores and presporogonic stages found both within and between myocytes appeared to primarily be a *post mortem* effect given that this was more frequently observed in fish dissected on the day following sampling. However, the presence of macrophages within myocytes having loose stages from a small number of fish that were dissected within hours of capture suggests that this event may occur at a low level within living fish. Cyst walls are known to dissolve in bullseye puffer *Sphoeroides annulantus* (Jenyns) infected with *Kudoa dianae* and spores travel through the esophageal connective tissue without eliciting an immune response (Dykova, Avila & Fiala 2002). For *K. dianae* this observation is explained as an adaptation to shed spores through the digestive tract (Dykova et al. 2002). At this time it is not clear whether *K. thyrsites* stages can spread between muscle cells.

Stehr and Whitaker (1986) described evidence of myoliquefaction in myofibrils in close proximity to the parasite from tissues collected immediately following euthanization (Morado &

Sparks 1986). The same authors also report 'coagulative necrosis' of myocytes with the presence of phagocytes, also supporting that some degree of myomere breakdown occurs while the fish is alive. Occurrence of myoliquefaction in live hosts has been described, but is rare. Eiras, Júnior, Sampaio, Robaldo and Abreu (2007) reported severe live myoliquefaction, presumed to have led to mortality, in a few individuals of farmed South American flatfish *Paralichthys orbignyanus* (Valenciennes) due to infections caused by a *Myxobolus* species. Davies, Andrews, Upton and Matthews (1998) report myoliquefaction in gobies, presumably caused by a *Kudoa* sp., but also may have been caused by secondary invaders. *In vivo* effects appeared to be limited in our samples. Dissections from same day only showed mild degeneration within infected myocytes; only samples collected from heavily infected fish from day 2 had extensive tissue breakdown.

Infection dynamics

Onset of infection showed similarities at both farm sites as well as to previous trials (e.g. Moran and Kent 1999; Moran *et al.* 1999a). The first detections between May and June were within the seasonal infectivity window (April 25th to December 4th) identified by Moran and Kent (1999). Spring entry trials by Moran and Kent (1999) and Moran *et al.* (1999a) also detected infections within two months of entry. Our evidence of first infections (355 and 509 degree days) were similar to the six week detection (approximately 420 degree days) found by Moran *et al.* (1999a) by PCR in multiple tissue, including blood. Visually we were able to detect *K. thyrsites* in the muscle after 975 and 1,113, but not at 778 degree days, similar to the 1,000 degree days reported by Moran and Kent (1999), but not as early as Moran *et al.* (1999a) and Young and Jones (2005) where early stages of cysts were described around 600 - 630 degree days.

PCR detection of the parasite within blood samples, coinciding with the onset of infection at both farms, supports a similar pattern of infection as presented by Moran *et al.* (1999a). Migratory stages within teleost circulatory systems have been described in several myxozoans e.g. (Holzer *et al.* 2003; Bjork & Bartholomew 2009). At the HP farm, detection in blood did reoccur but not at the same prevalence as the initial detection, possibly indicating a reduction in new infections in older fish. Most of the fish having positive blood results after 2,265 degree days also had moderate to heavy infections with evidence of inflammation and

phagocytosis of spores and therefore at least some of these results may be attributable to late rather than early stages of infection.

The highest prevalence of infection at the HP farm was between 2,265 and 4,839 degree days (approximately 9 and 17 months post entry) and was nearly 100%. Moran and Kent (1999) showed a peak at 2,000 degree days followed by a decline, based on two sampling points from the subsequent 12 months. We also report a decline at the HP farm, but not until 19 months (5,329) degree days. The current observation is based on three sample points within a five month period and is supported by both increased sampling effort by PCR and decreased intensity counts. These observed declines could be a result of infection resolution, possibly due to the inflammatory response, or reduced detection due to dispersal of pseudocysts from muscle fibre hyperplasia as the fish grew (Higgins & Thorpe 1990). Analysis using stereology and counts of leading edges of pseudocysts from sections taken at multiple depths could be used to calculate the number of infections per unit volume and extrapolated per individual fish (West 2012), but were beyond the scope of this survey.

Fish at the LP farm did not reach the infection prevalence or intensities found at either HP farm or Strait of Georgia fish. Instead, muscle PCR prevalence fluctuated between 37 and 73 % after 2,000 degree days. While exposure is a probable explanation, and the dosages of infective stages have a documented impact on infection prevalence (Bjork & Bartholomew 2009; Hallett, Ray, Hurst, Holt, Buckles, Atkinson & Bartholomew 2012), other variables, such as temperature, have also been linked to increased effects of myxozoan infections of fish (Schmidt-Posthaus & Wahli 2015). The relationship between exposure and infection has not been studied in *K. thyrsites* due to a lack of methods to measure the infective stage in the environment. Assuming all individuals were eventually exposed, the stable prevalence at the LP farm could be explained by a population level equilibrium between acquiring and resolving infections. Note that Moran *et al.* (1999b) found that some fish maintained in freshwater following a two week exposure to infective seawater were infected for as long as 23 months. Therefore, once infected, at least some of the pseudocysts may remain encysted for the duration of the fish's lifespan. It is possible that the plateau in prevalence in the LP farm may be due to population level resistance to new infections after low levels of exposure. This acquired resistance could explain the apparent decrease in prevalence and intensity seen at the HP farm as well as the decreases observed by previous studies (Moran & Kent1999; Moran *et al.* 1999a). Resolution, followed by

decreased exposure during the second year seems unlikely given the similarities in infection dynamics, especially between the HP farm in the Discovery Islands in 2011 and the Georgia Strait during the mid-1990s. The decrease in prevalence of the putative migratory stages within the blood supports the argument of population level onset of resistance to new infections.

Observations of resistance against reinfection by myxozoan pathogens have been reported (Golomazou, Athanassopoulou, Karagouni, Tsagozis, Tsantilas & Vagianou 2006; Foott & Hedrick 1987). Interactions involving specific and non-specific humoral and cellular responses have been described from myxozoan infections in various teleosts but have not been thoroughly investigated (Sitjà-Bobadilla, Schmidt-Posthaus, Wahli, Holland & Secombes 2015). Acquired resistance against myxozoans, linked to a specific adaptive immune response, has been demonstrated in turbots *Psetta maxima* (L) infected with *Enteromyxum scophthalmi* (Sitjà-Bobadilla, Palenzuela, Riaza, Macias & Alvarez-Pellitero 2007). Alternatively, age specific infection susceptibility to myxozoan infections has been described in some freshwater host parasite relationships, but may specifically be related to post hatch stages (Sollid, Lorz, Stevens & Bartholomew 2003). In anadromous fish, the effects of smoltification can decrease immune function (Melingen, Stefansson, Berg & Wergeland 1995; Maule & Shreck 1987), but the impacts on fish myxozoan interactions has not been explored.

Identification of young compared to mature pseudocysts may help to identify whether infections are old, new, or mixed. Descriptions of cyst developmental stages compared to fish age in *Kudoa* infections of wild fish populations vary with fish hosts and populations. For example, Heiniger and Adlard (2012) speculate that *Kudoa leptacanthae* occurs as a recurring infection in *Zoramia* spp. based on the presence of both young immature and older mature plasmodia within members of the population. Similarly, Morado and Sparks (1986) found in Pacific Hake, *M. productus*, that presporogonic stages of both *K. thyrsites* and *K. panaformis* were higher in fish under 40 cm. But this study also showed that presporogonic stages were present in all age classes with older fish having an increased overall prevalence. In contrast, Langdon *et al*. (1992) reported a tendency for distinct populations of Mahi mahi, *C. hippurus*, to be infected with either sporulated or unsporulated *K. thyrsites*. Older infected tubesnout, *A. flavidus*, had larger cyst sizes than younger fish (Shaw et al. 1997), although this may reflect reduced growth constraints within smaller myocytes.

Regional variation in K. thyrsites prevalence

Regional variation in *K. thyrsites* infections of Atlantic salmon has been described in British Columbia with northern Vancouver Island and the West Coast having lower prevalence than most Discovery Island farms (Karreman *et al.* 2003; Morrison & MacWilliam 2010a). Our infection intensity was also higher in the Discovery Island farm but lower than fish from experimental trials in the Strait of Georgia (Funk *et al.* 2007; Jones, Forster, Liao & Ikonomou 2012). An epidemiological survey of farms in British Columbia identified a variety of trends correlated with infection related to environmental parameters, such as, distance from bottom, bottom slope, salinity, dissolved oxygen as well as stress inducing husbandry practices, such as, transport and stocking density (Karreman *et al.* 2003). As of yet, none of these trends have been methodically investigated. Interestingly, infection prevalence in wild hake sampled along the coast of British Colombia does not show a parallel regional distribution to infected farmed salmon. King, McFarlane, Jones, Gilmore and Abbott (2012) report a prevalence of 91.7 % in summer Queen Charlotte Strait stocks located near less unaffected farms. Using histology this study demonstrated prevalences ranging from 30.8 % to 91.7 % out of 95 Pacific hake caught in regions where farmed Atlantic salmon are less affected and 100 % prevalence in fish caught in the Strait of Georgia were infected (King et al. 2012). It is curious why northern populations of hake are more infected than farmed Atlantic salmon in the same region, but the migratory ranges of Hake populations are not well understood.

Although *K. thyrsites* is a globally dispersed parasite and found in many wild fish (Whipps & Kent 2006), reports in Atlantic salmon from other regions are limited. Infections have been documented from Atlantic salmon in France (*cf.* Harrell & Scott 1985), Galicia, Spain -- including an anecdotal reference to other infections (Barja & Toranzo 1993), Ireland (Palmer 1995; Palmer 1995 cf. Whipps & Kent 2006), Chile (Lopez & Navarro 2000) and Tasmania (Munday, Su & Harshbarger 1998). Munday *et al.* (1998) provided an anecdotal report of regional variation in Atlantic salmon in Tasmania, which they suggest could be due to differences in antifouling practices or feed. Regional variation in other affected farmed fish, such as Mahi mahi, has not been assessed. Levsen *et al.* (2008) found *K. thyrsites* infections in wild Atlantic mackerel, *Scomber scombrus,* to be more common in stocks from Southwest of Britain than those from the North Sea and speculated that the mackerel may be acquiring their infections from the warmer areas of their distribution. Plausible environmental variables include preferable

habitat for the invertebrate host(s), water temperature, or host stressors. Alternately some regions may have more virulent strains.

Conclusions

Infection prevalence and intensity varied between two infected sites with one site having lower levels of *K. thyrsites* by all measures, yet populations at both sites had similarities in the overall presentation of infection. Infections under commercial conditions, especially at the high prevalence site, corresponded with observations of onset and development of experimental trials conducted 12 years prior at a nearby research facility. Both populations became infected around 500 degree days. Our high prevalence site reached 100 % saturation within 2,265 degree days and did not show any indication of resolution until the final six months while the LP site prevalence, once infected, plateaued around 50 % for the duration of the study. PCR detection and pseudocyst counts decreased over time but fillet manifestation remained constant. Detection in the blood occurred near the beginning of infection and again during the second year at sea. The initial detection in blood at both sites may indicate the presence of a blood borne stage. The overall patterns of infection provide circumstantial evidence for changes in susceptibility to new infections over time. Further work characterizing pseudocysts by size or development in chronically exposed fish or detection of specific antibodies could help determine whether fish develop immunity.

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Figure Legends

Figure 1. Muscle tissue sites selected for Atlantic salmon sample collection. Samples from the left and right side of fish are labeled as A, B, C, D and E, F, G, H, respectively.

Figure 2. Frequency histograms showing the percentage of fillets belonging to each **'K' (***Kudoa thyrsites***)** category at high (n=353) and low (n=181) prevalence farms throughout the entire fillet sampling period.

Figure 3. Percentage of type two error (false negatives) based on cumulative PCR results with increasing numbers of tested tissue samples. Values are based on PCR results from 19 samplings of 30 fish from the high prevalence farm. Fish were considered true negatives for *Kudoa thyrsites* if all eight tissue sites were PCR negative. Bars represent standard error over 19 samplings.

Figure 4. Prevalence of infection of *Kudoa thyrsites* by degree day at high and low prevalence farms based on PCR analysis of four tissue sites per fish. N = 30 fish per sampling, except 15 fish at 355 degree days at the LP farm.

Figure 5. Mean intensity of infection of *Kudoa thyrsites* at HP and LP farms over increasing degree days post entry measured as the number of pseudocysts counted per mm^2 from cross sections of up to 10 randomly chosen PCR positive fish per sampling time. N = 147 and 109 for each farm, respectively. Bars represent standard error.

Figure 6. Early development of pseudocysts (**A-D**) and putative migratory parasite cells (**E-H**). **A-B**. Young pseudocysts containing mono- and bi-nucleate cells (arrows). **C**. Cross section of a developing pseudocyst microvilli-like extensions (arrowheads) and mature spores with refractile polar capsules (small arrows) near center and undifferentiated cells near exterior. Note deeply stained pycnotic nuclei of capsulogenic cells to left of asterisks **D**. Longitudinal section of a developing pseudocyst filled mostly with undifferentiated cells, but also containing young spores with non-refractile, eosinophilic polar capsules. Myxospores are marked with asterisks and

arrowheads point to the interface between the edge of the pseudocyst and the host myocyte. **E**. Unidentified and seemingly multinucleate cell underlying dermal tissue (arrow) of a heavily infected fish at 1,676 degree days. **F**. Unidentified multinucleate cell within blood vessel (arrow) of a heavily infected fish at 1,113 degree days. **G**. Multinucleate cell (arrow) with resemblance to undifferentiated cells typically within pseudocysts. **H**. Unidentified multinucleate cell (arrow) in intracellular space between myocytes from a heavily infected fish at 1,113 degree days. A-B, E-H are Giemsa stained and C-D are H&E stained. Scale bars = 10 µm.

Figure 7. Ongoing development of pseudocysts, cellular host response and myoliquefaction. **A**. Pseudocyst localized within myocyte of a fish sampled at 2,013 degree days. Note undifferentiated cells along periphery. **B**. Large pseudocyst from a fish sampled at 6,389 degree days. Note the absence of undifferentiated cells and the presence of fibrous eosinophilic material (asterisks) between mature spores with pale mauve refractile polar capsules and deeply stained pycnotic capsulogenic nuclei. **C**. Inflammatory response in a fish at 1,676 degree days with visible spores (arrows). Inset (**D**) shows an engulfed spore within a macrophage. **E**. Pseudocyst has lost its integrity and spores and undifferentiated cells (arrow) are loose within the myocyte. Note that myofibrils are degenerating. This sample was taken 1 day post mortem from a fish sampled at 1,676 degree days. All material is Giemsa stained except figure B. Scale bars = 50 µm (A, B); 20 µm (C); and 10 µm (D, E).

Tables and Figures

Table 1. Sampling schedule, environmental records, biometry data of Atlantic salmon, and samples and procedures to detect *Kudoa thyrsites* from the high prevalence farm. Fish were entered on April 3rd, 2010.

Date	15-Apr-10	31-May-10	28-Jun-10	29-Jul-10	26-Aug-10	23-Sep-10	28-Oct-10	25-Nov-10	15-Dec-10	20-Jan-11	17-Feb-11	21-Mar-11	14-Apr-11	30-May-11	06-Jul-11	08-Aug-11	29-Sep-11	21-Nov-11	02-Feb-12	12-Apr-12
Days After Entry	12	58	86	117	145	173	208	237	257	292	320	352	376	422	459	492	544	597	670	740
Degree Days	105	509	778	1113	1400	1676	2013	2265	2437	2699	2901	3124	3300	3675	4017	4339	4839	5329	5884	6389
Average Monthly Temperature (ºC)	8.53	9.20	9.78	10.55	10.17	9.84	9.46	8.72	7.80	7.34	7.06	7.06	7.59	8.43	9.71	9.92	9.68	8.36	7.15	7.62
Average Monthly Dissolved Oxygen (mg/L)	8.45	8.21	7.32	7.24	6.14	5.63	5.44	6.05	7.03	7.07	7.23	7.39	7.21	7.51	6.01	5.69	5.26	5.59	6.95	9.31
Average Monthly salinity (‰)	29.95	29.97	30.00	29.75	29.26	28.68	29.49	29.68	29.89	30.00	29.97	29.82	29.89	29.97	30.00	30.00	29.68	29.89	29.96	30.00
Average Weight (g)		150	211	285	452	618	792	1032	1089	1285	1319	1723	1720	2227	2638	3481	4431	4513	6485	8746
Average Length (mm)		245	270	306	403	387	415	444	464	490	501	552	553	604	651	685	742	729	808	879
# Fish Sampled	15	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Muscle PCR: *Sampling effort	15	119	92	71	60	67	58	38	50	52	38	40	41	45	47	62	58	81	68	86
# Fish for histology from:																				
ºMuscle PCR-positive fish	0	0	10	10	10	10	10	10	10	10	10	10	10	10		10		8		9
ºAdditional fish (Blood PCR positive, additional K0-K2, or K4-K6)			6	1				1		1	6	2	7	1	9	6	3	2	2	8
# of fish having fillets analyzed							4	16	19	24	22	28	27	30	30	30	30	30	30	30

*Sampling effort calculated from a maximum of four tissue spots from the left side of 30 fish per month (120/month). Note that only one sample was collected from 15 fish during the first sample point in April 2010.

Table 2. Sampling schedule, environmental records, biometry data of Atlantic salmon, and samples and procedures to detect *Kudoa thyrsites* from the low prevalence farm (H hatchery; 1 first sea-site; and 2 second sea-site). Fish were entered on May 16th 2010.

Date	28-Apr-10	23-Jun-10	20-Jul-10	24-Aug-10	20-Sep-10	20-Oct-10	22-Nov-10	13-Dec-10	12-Jan-11	21-Feb-11	28-Mar-11	11-Apr-11	16-May-11	20-Jun-11	25-Jul-11	29-Aug-11	24-Oct-11	09-Jan-12	27-Mar-12
Site	H	1	1	1	1	1	1	1	1	2	2	2	2	2	2	2	2	2	2
Days After Entry	0	37	65	100	127	157	190	211	241	281	316	330	365	400	435	470	526	603	681
Degree Days	0	355	622	975	1236	1518	1804	1962	2176	2448	2682	2783	3053	3369	3708	4068	4584	5172	5694
Average Monthly Temperature (ºC)		9.67	10.00	10.07	9.40	9.18	7.99	7.39	6.83	6.68	6.85	7.30	8.54	9.47	9.69	10.05	9.00	6.80	7.06
Average Monthly Dissolved Oxygen (mg/L)		9.26	8.38	7.10	6.08	6.84	7.88	7.95	8.23	8.87	9.42	9.21	9.65	9.45	8.23	7.18	7.35	6.64	8.59
Average Monthly salinity (ppt)		33.14	34.00	34.00	34.00	34.00	34.00	34.00	33.97	34.00	34.00	34.00	34.00	33.57	33.28	33.57	33.09	30.86	33.00
Average Weight (g)	43.9	122		149	244	359	468	410	475	718	783	893	1063	1240	1850	2319	2045	3632	5230
Average Length (mm)	163	225	210	241	281	315	354	343	356	411	429	444	470	498	565	592	580	676	759
# Fish Sampled	30	15	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
Muscle PCR: *Sampling effort	30	60	107	102	96	102	81	75	89	79	86	85	62	86	88	81	89	91	92
# Fish for histology from:																			
ºMuscle PCR-positive fish	0	0	0	4	4	3	10	10	9	10	10	9	8	9	0	9	6	0	6
ºAdditional fish (Blood PCR positive,)																1			
# of fish having fillets analyzed										2	5	9	15	0	30	30	30	30	30

*Sampling effort calculated from a maximum of four sites from the left side of 30 fish per month (120/month). Exceptions are the first two sample points where only one sample was analyzed per fish during the first sample point only 15 fish were collected in June.

** Pseudocyst densities were also measured for all blood PCR positive fish.

Table 3. Nested PCR primer sequences

Amplification	**Sequence 5'-3'**	
First	Forward	AGA AAT ACC GGA GTA GAC CGT
	Reverse	TGA TCG TCT TCG AAC CTC CT
Second	Forward	CGT AGT TGG ATT ACA AAA GCT CTG
	Reverse	GTT CCA TGC TAT AAC ATT CAA GC

Table 4. Histological observations in *Kudoa thrysites* PCR positive fish in blood (all) and in muscle (10 randomly selected, except * and ** with 8 and 9 fish) from the high prevalence Atlantic salmon farm.

	Blood PCR Positive Fish			Muscle PCR Positive Fish		
Degree Days	**PCR Positive Fish (no.)**	**Prevalence by Histology (%)**	**Fish with Inflammation (%)**	**Prevalence by Histology (%)**	**Fish with Inflammation (%)**	**Spores in Inflammatory Foci (%)**
788	7	0	-	0	0	0
1113	1	0	-	40	20	0
1400	0	-	-	100	40	10
1676	0	-	-	100	90	80
2013	0	-	-	100	60	30
2265	1	100	100	90	90	60
2437	0	-	-	90	80	40
2699	1	100	100	90	80	30
2901	1	100	100	90	70	10
3124	2	100	50	80	50	30
3300	5	80	80	90	70	40
3675	4	100	50	50	20	10
4017	4	100	100	-	-	-
4339	1	100	100	80	60	20
4839	3	67	33	-	-	-
5329	0	-	-	88*	0	0
5884	2	100	100	-	-	-
6389	1	0	-	67**	30	10

Table 5. Corresponding histological observations on blood positive fish and randomly selected PCR positive fish sampled from the low prevalence site.

Degree Days	Blood PCR Positive Fish		Muscle PCR Positive Fish			
	PCR Positive Fish (no.)	*Prevalence by Histology %	PCR Positive Fish (no. examined)	Prevalence by Histology (%)	Fish with Inflammation (%)	Spores in Inflammatory Foci (%)
975	2	50	4	50	0	0
1236	0	-	4	100	0	0
1518	0	-	3	100	33	0
1804	0	-	10	100	60	20
1962	0	-	10	90	60	30
2176	0	-	9	67	44	11
2448	0	-	10	60	60	10
2682	1	100	10	50	40	20
2783	1	100	9	67	67	33
3053	0	-	10	50	20	0
3369	1	100	9	44	33	0
4068	2	50	9	44	33	0
4584	0	-	6	33	33	17
5694	0	-	6	33	67	0

*No inflammation was observed on blood positive fish.

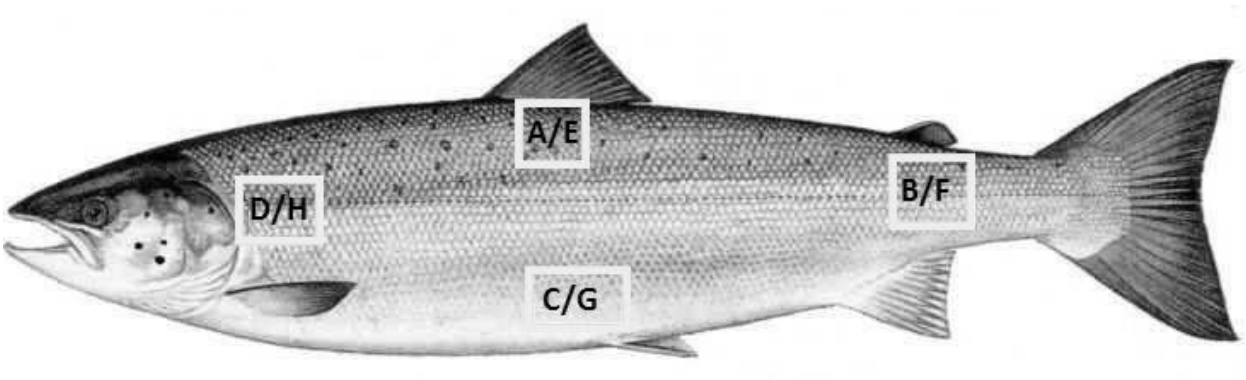

Figure 1.

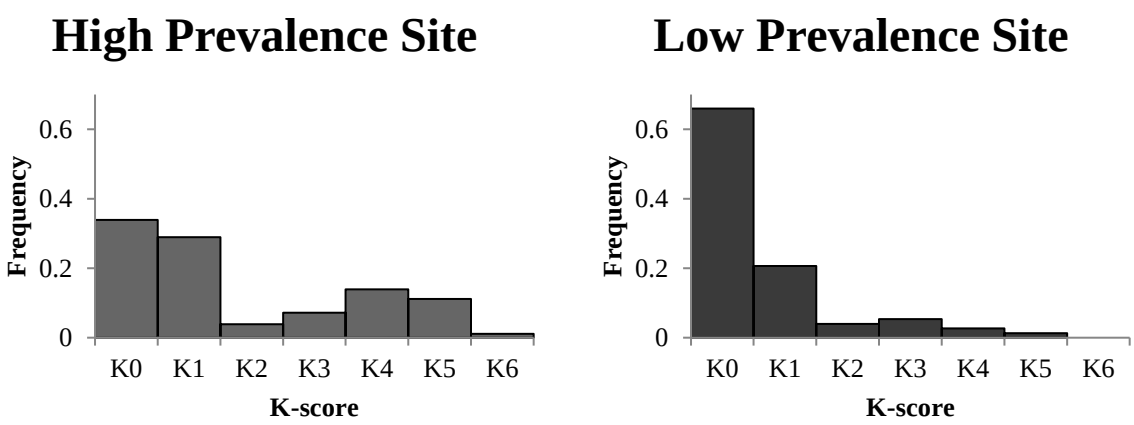
High Prevalence Site
Low Prevalence Site
Frequency
0.6
0.4
0.2
0
K0
K1
K2
K3
K4
K5
K6
K-score

Figure 2.

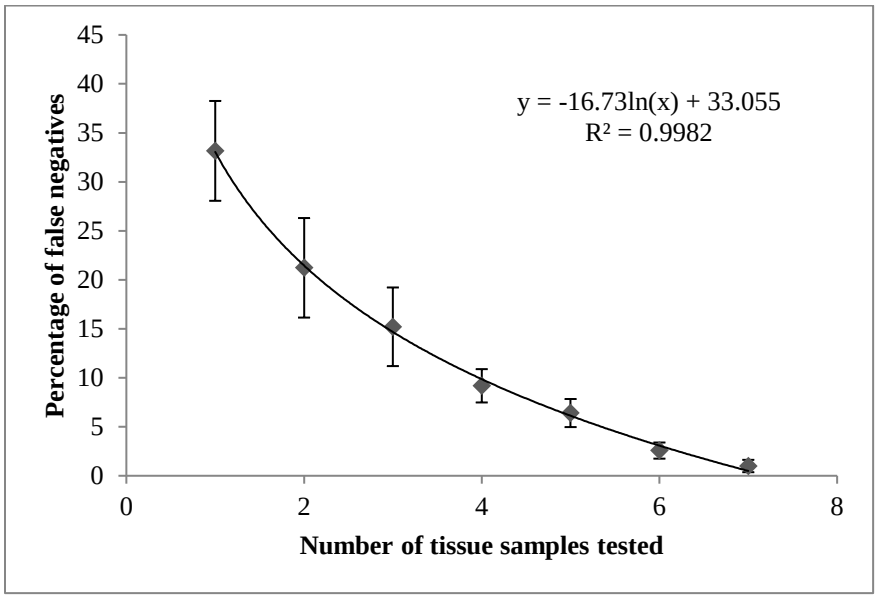
y = -16.73ln(x) + 33.055
R² = 0.9982
Percentage of false negatives
Number of tissue samples tested
45
40
35
30
25
20
15
10
5
0
0
2
4
6
8

Figure 3.

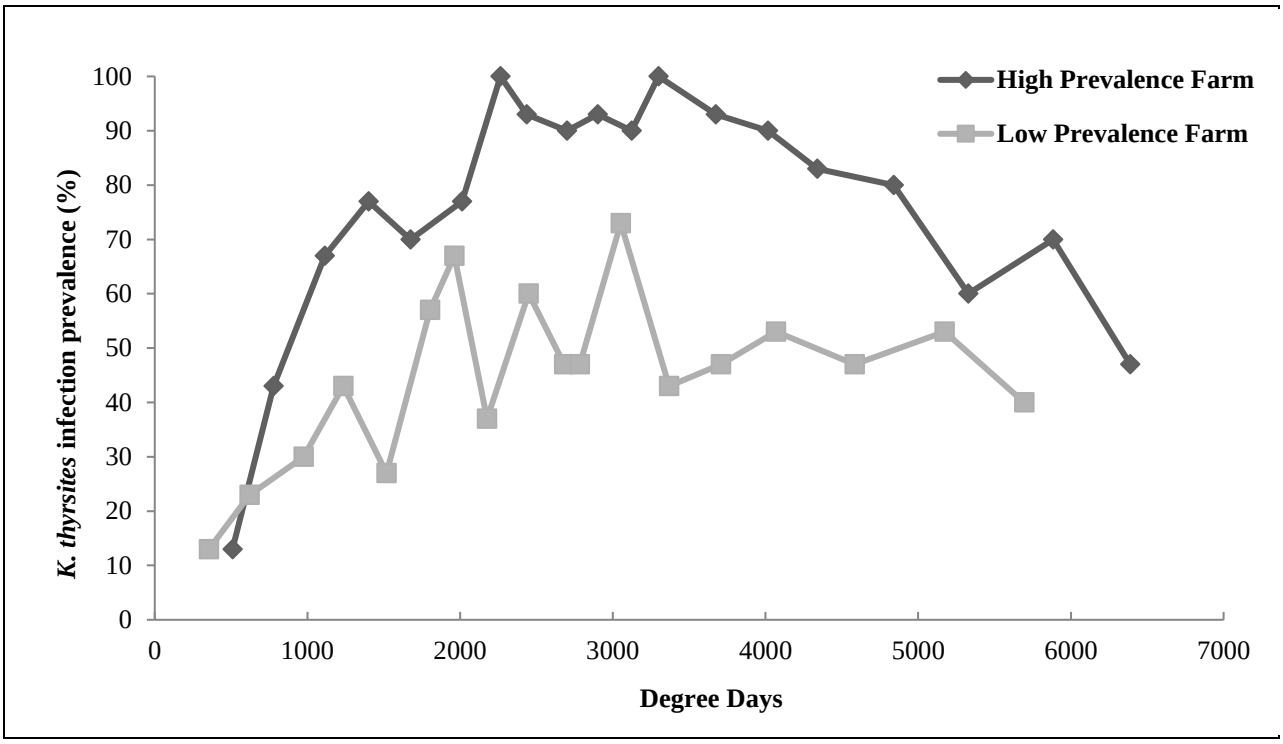
High Prevalence Farm
Low Prevalence Farm
K. thyrsites infection prevalence (%)
Degree Days
0
10
20
30
40
50
60
70
80
90
100
0
1000
2000
3000
4000
5000
6000
7000

Figure 4.

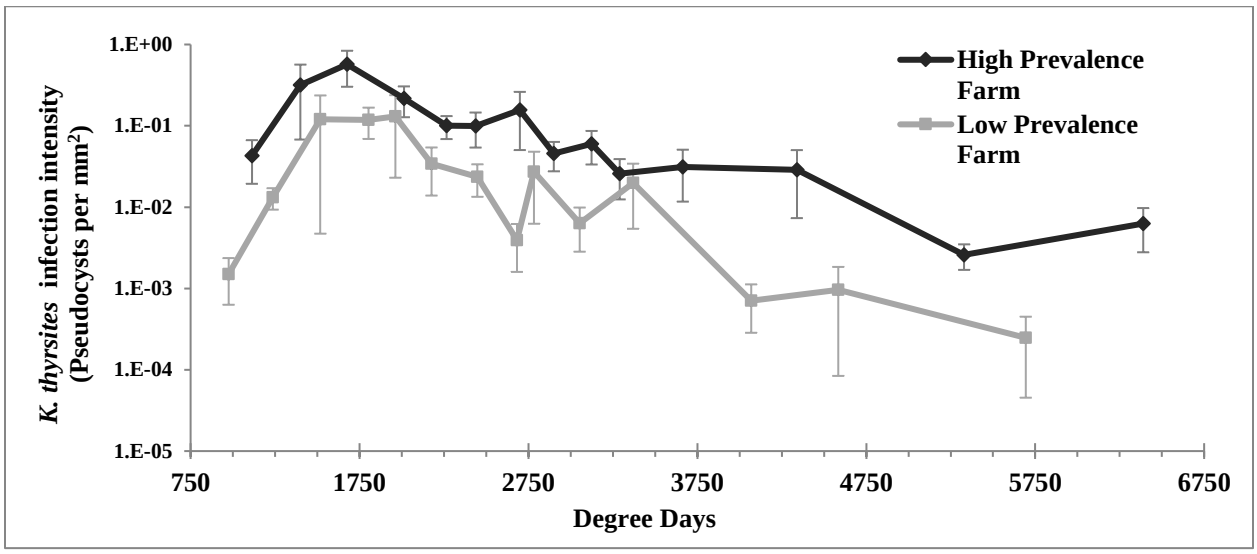
1.E+00
1.E-01
1.E-02
1.E-03
1.E-04
1.E-05
K. thyrsites infection intensity (Pseudocysts per mm2)
750
1750
2750
3750
4750
5750
6750
Degree Days
High Prevalence Farm
Low Prevalence Farm

Figure 5.

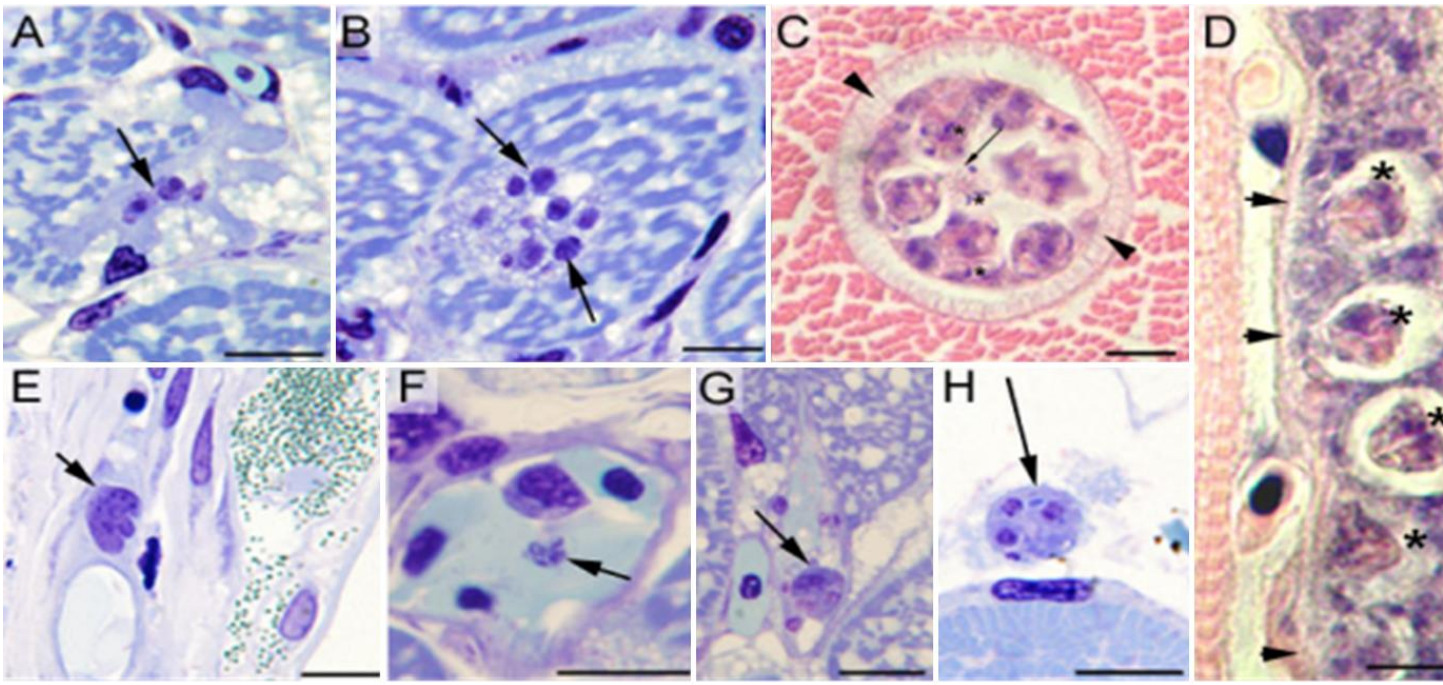

Figure 6.

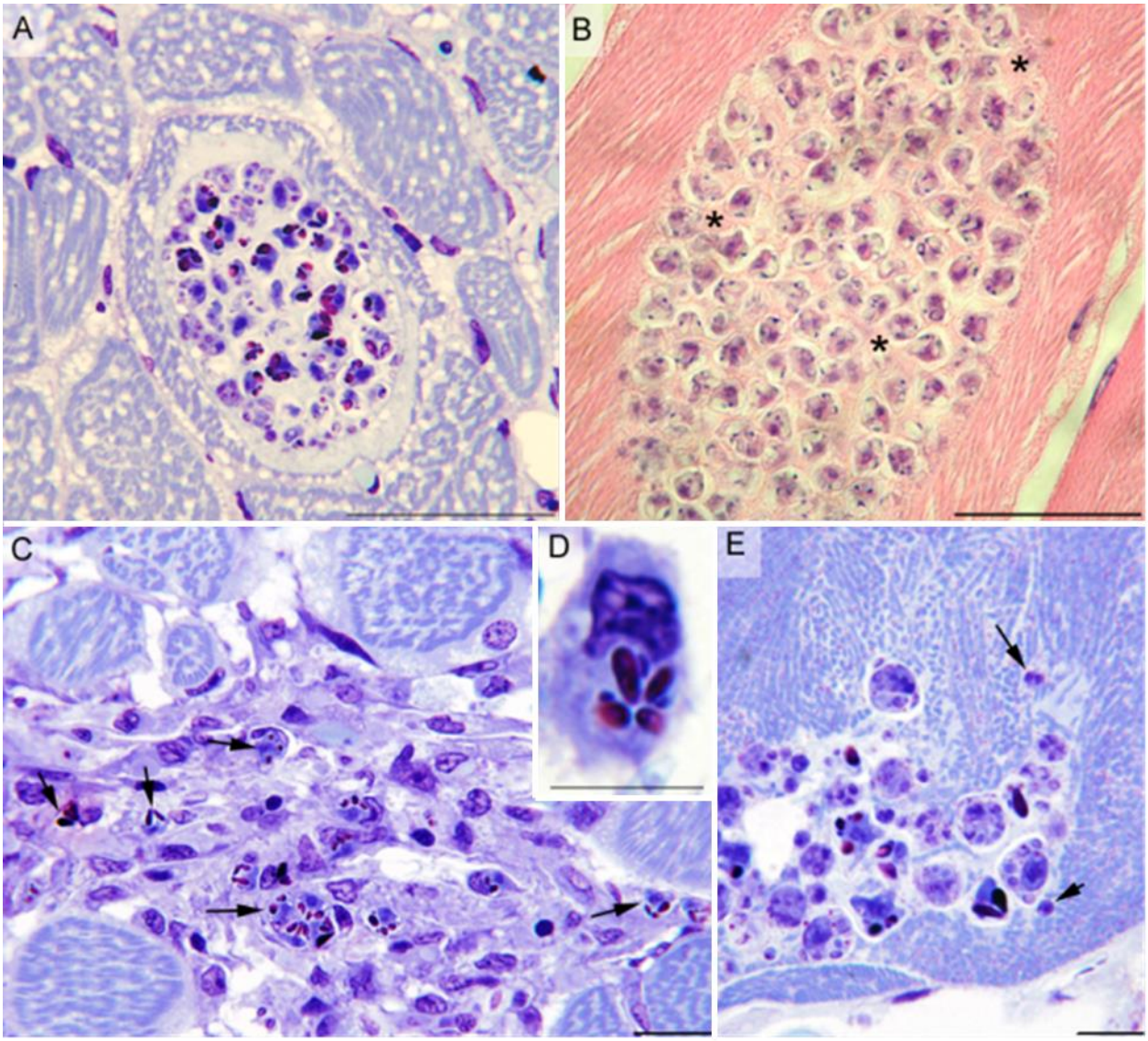

Figure 7.

Supplementary Files

Table S1. Fillet manifestation categories due to *Kudoa thyrsites* from Atlantic salmon fillets collected at sampling times having 27 or more fish greater than 1 kg from each farm.

Site	High Prevalence									Low Prevalence				
Sampling Date	21-Mar 2011	14-Apr 2011	30-May 2011	06-Jul 2011	08-Aug 2011	29-Sept 2011	21-Nov 2011	02-Feb 2012	12-Apr 2012	25-Jul 2011	29-Aug 2011	24-Oct 2011	09-Jan 2012	27-Mar 2012
Fillets Collected (#)	28	27	30	30	30	30	30	30	30	30	30	30	30	30
K0: no pitting (%)	21	19	13	43	33	33	43	27	23	70	90	63	40	67
K1: 1-10 pits (%)	43	33	40	17	30	33	27	33	33	17	7	20	37	23
K2: 11-20 pits (%)	4	11	13	7	7	0	0	7	3	0	0	10	7	3
K3: 21-40 pits (%)	7	0	7	10	3	10	13	3	3	3	3	0	13	7
K4: >40 pits (%)	14	15	7	7	7	7	13	20	30	7	0	3	3	0
K5: severe pitting (%)	11	19	20	13	17	17	3	10	7	3	0	3	0	0
K6: liquefied (%)	0	4	0	3	3	0	0	0	0	0	0	0	0	0

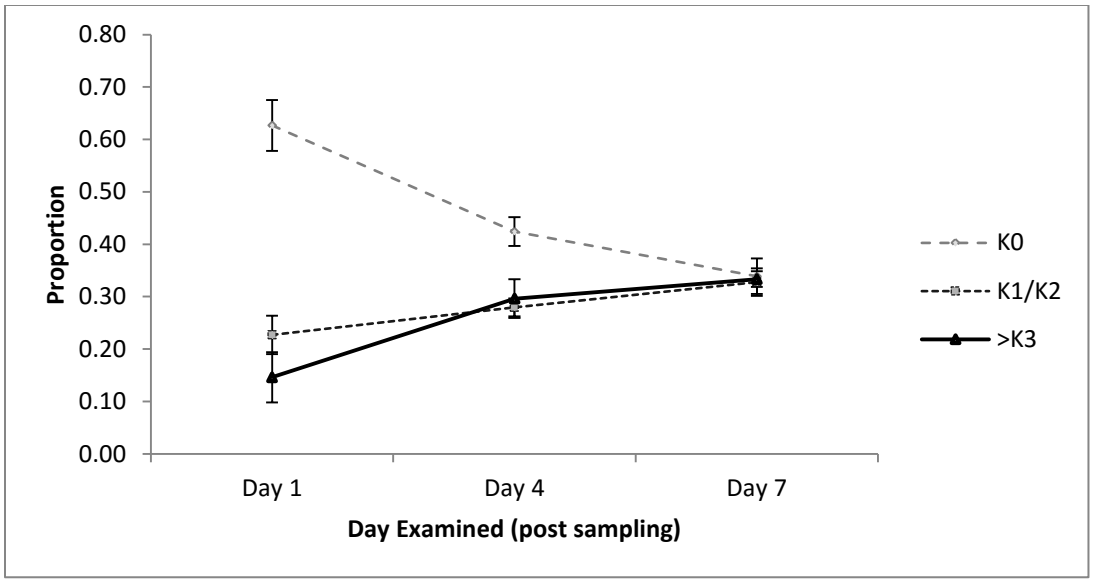

Figure S1. Proportion of fillets belonging to unaffected (K0), lightly infected (K1/K2), or heavily infected (>K3) *Kudoa thrysites* categories according to the number of days after sampling. Averages were calculated from all fish analyzed within the final six sampling times at the high prevalence Atlantic salmon farm (n = 180 fillets). Bars represent standard error.

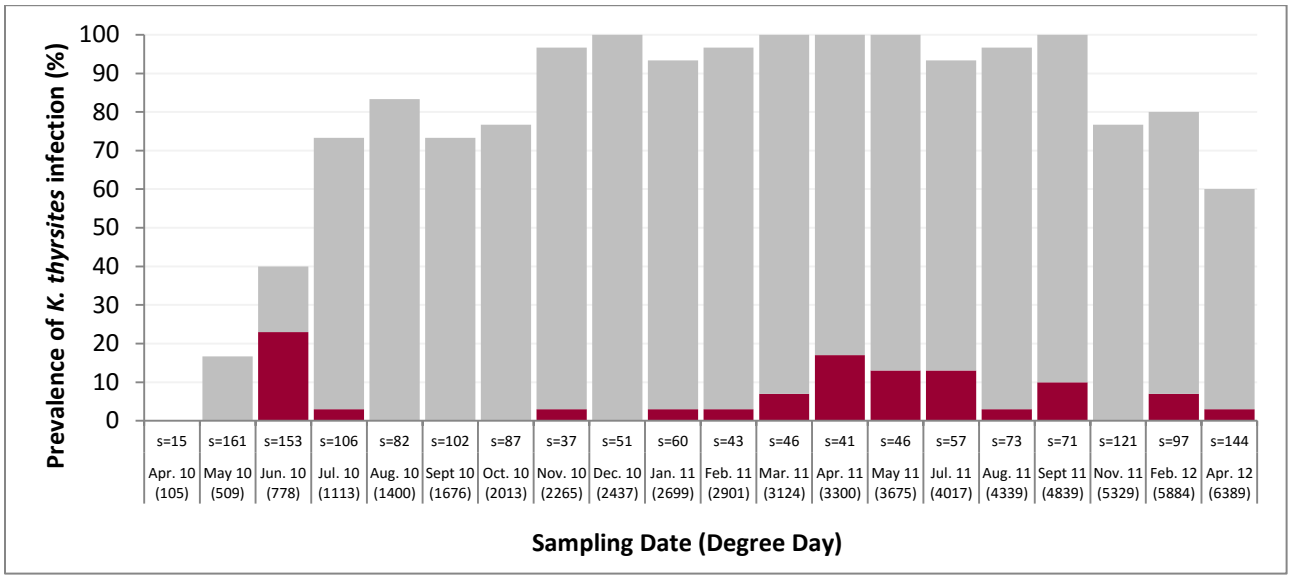

Figure S2. Prevalence of *Kudoa thyrsites* in muscle (grey) and blood (red) samples collected from the high prevalence Atlantic salmon farm as determined by nested PCR. Muscle infections for all dates (degree days), except for April 2010 (105 degree days), are based on eight muscle sites from 30 fish per sample (n=15 for April 2010). Blood was not sampled at 105 degree days. `s` refers to the sampling effort calculated as the number of PCR reactions on muscle each month.

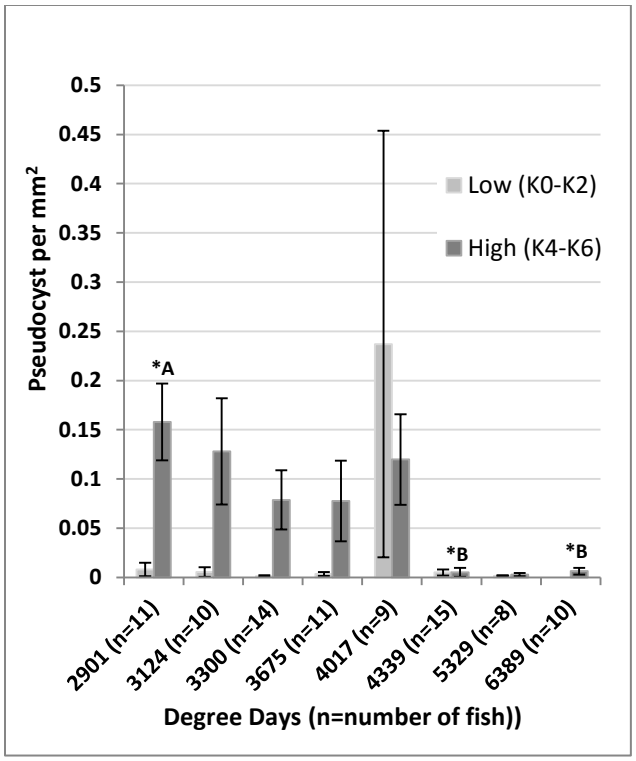

Figure S3. Mean number of pseudocysts of *Kudoa thyrsites* per mm^2 from fish belonging to high (K4-K6) or low (K0-K2) fillet manifestation categories with increasing degree days. Bars represent standard error of the mean and asterisks denote degree days with significant pairwise difference in pseudocyst counts from fish with high manifestation levels (A) compared to those with low levels (B) (Kruskal-Wallis AOV).