Published in final edited form as:

Fish Shellfish Immunol. 2014 March ; 37(1): 87–95. doi:10.1016/j.fsi.2013.12.024.

Defenses of Susceptible and Resistant Chinook Salmon (*Onchorhynchus tshawytscha*) Against the Myxozoan Parasite *Ceratomyxa shasta*

Sarah J. Bjork^a, Yong-An Zhang^{b,c}, Charlene N. Hurst^a, Maria E. Alonso-Naveiro^d, Julie D. Alexander^a, J. Oriol Sunyer^c, and Jerri L. Bartholomew^{a,*}

^aDepartment of Microbiology, Oregon State University, Corvallis, OR, USA, 97331

^bState Key Laboratory of Freshwater Ecology & Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, Hubei China 430072

^cDepartment of Pathobiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia, PA, USA 19104

^dInstituto de Acuicultura Torre de la Sal, Consejo Superior de Investigaciones Científicas (IATS-CSIC), Castellón, Spain 12595

Abstract

We investigated intra-specific variation in the response of salmon to infection with the myxozoan *Ceratomyxa shasta* by comparing the progress of parasite infection and measures of host immune response in susceptible and resistant Chinook salmon *Oncorhynchus tshawytscha* at days 12, 25 and 90 post exposure. There were no differences in invasion of the gills indicating that resistance does not occur at the site of entry. In the intestine on day 12, infection intensity and Ig+ cell numbers were higher in susceptible than resistant fish, but histological examination that timepoint showed more severe inflammation in resistant fish. This suggests a role for the immune response in resistant fish that eliminates some parasites prior to or soon after reaching the intestine. Susceptible fish had a higher IFN γ , IL-6 and IL-10 response at day 12, but all died fatal enteronecrosis by day 25. The greatest fold change in IFN γ expression was detected at day 25 in resistant Chinook. In addition, the number of Ig+ cells in resistant Chinook also increased by day 25. By day 90, resistant Chinook had resolved the inflammation, cytokine expression had decreased and Ig+ cell numbers were similar to uninfected controls. Thus, it appears that the susceptible strain was incapable of containing or eliminating *C. shasta* but resistant fish: 1) reduced infection intensity during early intestinal infection 2) elicited an effective inflammatory response in the intestine that eliminated *C. shasta* 3) resolved the inflammation and recovered from infection.

© 2014 Elsevier Ltd. All rights reserved.

*Corresponding author. Tel.: +1-541-737-1856; fax: +1-541-737-0496 bartholj@science.oregonstate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

Chinook salmon; resistance; immune response; inflammation; cytokine; Myxozoa

1. Introduction

Resistance of salmonids to the myxozoan parasite *Ceratomyxa shasta* is an inherited trait [1, 2, 3] conferred through unique genetic loci [4], but the mechanism is unknown. Although strains of native fish sympatric with the parasite are considered “resistant” [1, 2, 5, 6, 7, 8, 9], they may become infected and succumb to disease at high parasite dose [10, 11, 12, 13] and high water temperatures [14, 15]. Researchers investigating the mechanisms of the fish host response proposed three defense strategies against *C. shasta*: resistance against parasite entry and establishment, the mounting of an effective immune response that contains and/or eliminates the parasite, and parasite tolerance [8, 9, 16, 17]. In the years since the suggestion of these strategies, researchers have solved the *C. shasta* life cycle and developed a laboratory model for infection [18, 19], identified the route of parasite invasion and migration [19], determined threshold parasite doses [10, 13, 20, 21], and uncovered relationships between parasite genotype and pathogenicity in the salmonid host [22, 23]. These advances permit a re-examination of the above hypotheses to account for unknown parasite dose, course of infection and parasite genotype.

The complex life cycle of *C. shasta* involves two hosts; a salmonid and a freshwater polychaete, *Manayunkia speciosa*. The infectious stage of the parasite, the actinospore, is released from the polychaete and attaches to the gills of a fish. In the initial stages of infection, the parasite invades the gill epithelium and enters the bloodstream, a site of proliferation and means of transport to the intestine [19]. As infection progresses, parasites migrate through the serosa and lamina propria and proliferate between mucosal epithelial cells of the intestine, culminating in myxospore maturation. In severe infections, lymphocytes infiltrate the tissue and the intestine becomes grossly enlarged, inflamed and necrotic, resulting in enteronecrosis (ceratomyxosis). Parasites may occlude the intestinal lumen and pathological changes coinciding with parasite proliferation can occur in other organs [17]. Based on current knowledge of the route of actinospore invasion and migration, we modify the first defense strategy hypothesis to: resistance against parasite entry and establishment at the gills and/or intestine.

Conclusions made concerning defense strategies from previous studies are obscured by researchers’ reliance on field experiments for parasite infection. In these cases, parasite dose and genotype were unknown and/or could not be controlled. In lethal infections of resistant Chinook salmon (*Oncorhynchus tshawytscha*) there was no evidence of parasite exclusion or containment in the gills or in the intestine [19]. Increases in plasma lysozyme, complement and phagocytosis were demonstrated in lethal infections; however, these innate defenses failed to provide protection against disease when challenged with a high parasite dose [13]. In sub-lethal *C. shasta* infections of unknown genotype in resistant rainbow trout (*O. mykiss*), the parasite was contained within granulomata [8] and in resistant steelhead (*O. mykiss*), parasites were detected only in the lumen of the intestine [16, 17]. In susceptible

rainbow trout that survived a low dose of a presumably non-pathogenic parasite genotype (1 parasite per fish of some genotypes are lethal [20]), parasites were also found in the lumen and there was evidence of antibody production [24]. These studies suggest two very different host responses; one in which the parasite is contained prior to sporulation (as typically occurs in the lamina propria) and the other in which the parasite progresses through the lamina propria to the intestinal lumen without extensive inflammation or necrosis (tolerance). The variation in responses could be attributed to differing parasite doses or reflect unique responses to specific parasite genotypes. Thus, examination of defense strategies requires the ability to conduct sub-lethal challenges with controlled parasite genotypes.

The goal of this study was to identify the strategies resistant salmonids have evolved to survive in sympatry with *C. shasta*. To minimize variables such as species, genotype, and parasite dose, comparisons are made between resistant and susceptible strains of Chinook salmon to infection by genotype I, a pathogenic genotype for Chinook salmon [22, 23]. To determine the potential location and timing of parasite containment or elimination we quantified parasite DNA in the gills and examined histological sections taken immediately after infection. At three times after challenge we scored infection intensity and cellular responses using histology, evaluated antibody response by immunohistochemistry, and measured expression of pro-inflammatory and regulatory cytokines to identify fish strain-specific and temporal differences in the regulation of the immune response over the course of an experimental infection.

2. Materials and methods

2.1 Fish strains

Naïve fish of a susceptible (Salmon River (SR) Hatchery, OR, USA) and resistant (Iron Gate (IG) Hatchery, CA, USA) strain of Chinook salmon (SR average 7.0 ± 1.2 g; IG average 6.2 ± 1.2 g) were transferred to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis, OR. Fish were fed a daily commercial diet (Bio-Oregon, Longview, WA, USA) and reared in 13°C specific pathogen free (SPF) well water until initiation of the experiment.

2.2 Parasite source

As a source of actinospores, a culture of the invertebrate host *Manayunkia speciosa* was infected with myxospores obtained from Chinook salmon (genotype I) using methods previously described [19]. Water from the culture tank flowed into an aquarium where fish were held in separate cages segregated by strain for the parasite challenge. Three 1 L water samples were collected at the beginning of the fish challenge and parasite numbers were determined by quantitative PCR (qPCR) [25]. Water flow rate through the tank and parasite density measured by qPCR were used to estimate the total parasite dose for each experiment. Parasite exposure varied between the two experiments and is specified for each.

2.3 Resistance at the portal of entry (gills)

To compare *C. shasta* actinospore invasion in the gills, 10 fish of each strain were challenged simultaneously for 24 h. Based on qPCR estimates and flow rate, these fish were exposed to 1.7×10^4 actinospores fish⁻¹, below the estimated lethal threshold of 3.8×10^4 actinospores [13, 21]. After 24 h, 5 fish from each strain were euthanized with an overdose of MS-222 (tricaine methosulfonate, Argent, Redmond, WA) and the gills from one side of the head of each fish were fixed for histology in Davidson's fixative for 24 h then transferred to 70% ethanol. To assess parasite density, the other gill set was frozen for assay by qPCR. The remaining 5 fish from each strain were transferred to 13 °C SPF flow-through 25 L tanks to monitor the development of infection over 60 days. As a control, 5 uninfected fish from each strain were maintained identically, but in separate tanks. Because of the limited availability of these fish, gills were not collected for qPCR from control fish at 24 h, but these fish were assessed for the presence of *C. shasta* infection in the intestine by PCR [26, 27] at the end of the experiment.

DNA was extracted from gill tissue using the Qiagen DNeasy Blood and Tissue kit® (Valencia, CA, USA), eluted in 60 µL of AE buffer, then re-applied to the column and eluted again to increase yield. The DNA was then assayed by qPCR [25]. Samples were run in duplicate and Cq (quantification cycle) values were averaged for each sample. Two positive controls (DNA from infected fish tissue and synthetic *C. shasta* DNA template) and a negative control of molecular grade water were included. If the standard deviation of the duplicates was greater than 1, the samples were re-run. Samples in which parasite DNA was not detected were assigned a Cq of 40 to facilitate data analysis. A Student's *t*-test was performed to determine if there were significant differences in the Cq values between strains using SAS v. 9.3 (SAS Institute, Cary, NC, USA).

Histological sections were prepared by the Veterinary Diagnostic Laboratory, Oregon State University (OSU) Corvallis, OR, and stained with May-Grunwald Giemsa. The presence and location of the parasite within the gill tissue were compared between strains.

2.4 Comparison of histopathology and cytokine expression

2.4.1 Sample collection—In a second experiment, IG and SR strains of Chinook salmon were challenged with a dose of 4.3×10^3 actinospores fish⁻¹ in the exposure tanks. Thirty fish of each strain were placed in separate 40 × 15 cm cylindrical cages and held in a 136 L flow-through aquarium receiving water from the outflow of the infected polychaete colony for 24 h. An equal number of fish from each strain were held in cages in UV treated Willamette River water at the same flow rate for 24 h as a negative control. After exposure each treatment (IG infected (IGI), IG control (IGC), SR infected (SRI), SR control (SRC)), was divided into 3, 25 L SPF flow-through tanks at 13 °C with 10 fish per tank. Nine fish from each treatment (3 per replicate) were sampled 12, 25 and 90 days after exposure.

Fish were euthanized with an overdose of MS-222 and intestinal samples were collected to compare histopathology and cytokine expression. A section of the posterior intestine (30 mg, approximately 0.5 cm) was excised, preserved in RNALater (Qiagen) and stored at -80

°C until RNA extraction. The remainder of the intestine from each fish was preserved for histology as described in section 2.3.

2.4.2 Histopathology processing and analyses—One transverse section per fish was stained with Giemsa to determine infection intensity and another with H&E for inflammation. The entire length of the intestine was examined at 200× magnification. Infection intensity, in terms of number of parasite foci (one or greater parasites clustered in a group), was scored on a scale of 0–5: 0 = no foci, 1 = 1–5 foci, 2 = 6–10 foci, 3 = 11–15 foci, 4 = 16–20 foci, 5 = >20 foci. Inflammation was scored similarly: 0 = no foci, 1 = 1–10 foci, 2 = 11–20 foci, 3 = 21–30 foci, 4 = > 30 foci. In areas of confluent inflammation, each villi where inflammation was present was considered a single focus of inflammation. For statistical analyses, comparisons between infection intensity and inflammation between strain and treatment on day 12, and treatment and day for only the IG fish were conducted using two separate Kruskal-Wallis tests followed by Wilcoxon two-sample tests for pairwise comparisons. Comparisons were not conducted in SR fish after day 12 because all fish had succumbed to infection 24 days post exposure.

2.4.3 Immunohistochemistry—Identification of Ig⁺ cells was conducted by immunohistochemistry using a monoclonal antibody raised in mouse against Ig⁺ cells (undetermined Ig class) in rainbow trout [28] in a third set of sections. Slides were deparaffinated, rehydrated and blocked with 0.33% hydrogen peroxide for 30 min to eliminate endogenous peroxidase activity. Slides were then washed twice in 1xPBS for five minutes and autoclaved in 0.01M citrate buffer (pH 6) for 15 min at 121 °C for antigenic retrieval. After washing as before in 1xPBS, slides were blocked for non-specific binding using 1.5% goat serum (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) for 30 min in a 22 °C humid chamber. Slides were then incubated for 60 min with 0.02 mg/mL of the primary monoclonal biotinylated A3 antibody [28] in a 22 °C humid chamber. Following another 1xPBS wash, the slides were incubated with the ABC complex according to kit instructions, washed in 1xPBS, and the chromagen DAB (Sigma-Aldrich, St. Louis, MO, USA) was added. The DAB reaction was stopped with deionized water after 2 min and the slides were counterstained with 25% Gill's hematoxylin, dehydrated and mounted. Ig⁺ cells were counted using Image J software version 1.440 (NIH, <http://rsbweb.nih.gov/ij>) at 400×. Three fields per fish were counted and averaged. To examine differences in Ig⁺ cell counts among strain-day and treatment, we used a two-way ANOVA followed by Tukey's test for highly significant differences. Differences were considered significant at $p < 0.05$ using SAS v. 9.3 (SAS Institute).

2.4.4 Cytokine expression processing and analyses—For RNA extraction, intestinal tissue from individual fish was removed from RNALater and a ~30 mg piece was processed using the RNeasy Minikit® with in-column DNase I treatment (Qiagen). Total RNA concentration and purity were determined by Nanodrop. Prior to cDNA synthesis, RNA samples were either diluted with molecular grade water or concentrated using a Savant speed vac concentrator with refrigerated vapor trap to obtain 4 µg of RNA from the intestine for cDNA synthesis according to the Superscript™ First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, Carlsbad, CA, USA) using oligo dT primers.

The pro-inflammatory (IL-1 β , IL-2, TNF α , IL-6 and IFN γ) and regulatory (IL-10, and TGF β) cytokines were chosen to look for early responses to the parasite as well as regulation of the immune response. Primers for amplification of the housekeeping gene β -actin, as well as IL-1 β , IFN γ and TNF α were designed from Chinook salmon sequences in GenBank. Primers for amplification of IL-2, IL-6, IL-10, and TGF β transcripts were designed based on rainbow trout sequences in GenBank. Serial dilutions of a standard cDNA preparation were used to assess PCR efficiency. Primer concentrations, reaction efficiencies and GenBank accession numbers are listed in Table 1. The dissociation curves of the PCR products were analyzed to verify a single peak and products were separated by gel electrophoresis to corroborate the presence of only one product and to evaluate product size.

Quantitative PCR to measure gene expression was performed in 10 μ L reactions containing 5 μ L Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA, USA), 2 μ L molecular grade water, 0.5 μ L (concentrations in Table 1) of forward and reverse primer and 2 μ L of cDNA (diluted 1:10) in MicroAmp Fast Optical 96 well reaction plates. Each sample was run in duplicate through 40 cycles on an ABI 7500 Fast Real-time PCR system on the Standard 7500 setting with an added dissociation step and ROX as passive reference. Only samples for which both wells fluoresced were considered positive. If the standard deviation of the duplicate wells was >1 , the sample was re-run.

Cytokine expression was initially measured in infected and control treatments using 3 pooled samples comprised of 3 fish each (1 fish from each replicate tank per pool) for each strain on each sampling day. When differences were detected between treatment and control fish using *t*-tests on log transformed data, cytokine expression was quantified in individual fish. The log fold change in cytokine expression between the control and treatments were evaluated using REST 2009 software [29, 30] with 1000 randomizations. Differences between the control and treatments were considered significant at $p < 0.05$.

3. Results

3.1 Resistance at the portal of entry (gills)

Ceratomyxa shasta was detected by qPCR in 100% of the gills of both resistant and susceptible strains of Chinook salmon exposed in experiment 1. Cq values were not different between SRI (29.97 ± 1.7) and IGI (30.46 ± 0.77) treatments ($p = 0.56$). Histology revealed no differences in infection intensity or parasite location in the gills between strains; numbers of visible parasites were one or zero. Despite the low parasite numbers detected in the gills, all of the SRI held after the exposure succumbed to infection, with a mean day to death of 32.0 ± 9.0 . All of the IGI survived to 60 days and myxospores were not detected in wet mounts upon termination. All of the control fish were negative by PCR.

3.2 Histopathology

In the second experiment, infection in the SRI fish was fatal (mean day to death 22.0 ± 0.6), thus they were sampled only on day 12, while IGI cohorts survived to day 90. Therefore, measures of infection intensity, inflammation and numbers of Ig+ cells were compared

between strains only at day 12. These measures were compared within the IG strain at days 12, 25 and 90. Neither parasites nor inflammation were detected in any of the controls.

At day 12, infection intensity in the intestine was higher in SRI than IGI ($p = 0.001$), but inflammation was higher in IGI than SRI (Fig. 1a–b, $p = 0.0062$). SRI exhibited mild to moderate, multi-focal and locally extensive inflammation in the lamina propria, dominated by mononuclear cells with a few neutrophils (Fig. 2a–b). *C. shasta* trophozoites were observed in all layers of the posterior intestine (mucosal epithelium, lamina propria, muscularis, and serosa) from all nine of the SRI, but predominately in the mucosal epithelium (Fig. 3a–c). IGI exhibited moderate to diffuse lymphocytic enteritis (Fig. 2c–e), with trophozoites observed in eight of the nine fish (Fig. 3c–d). In three IGI, the mononuclear cell response was transmural, and scattered macrophages were present. Two of these also had increased vascularization in the lamina propria, evidenced by dilated blood vessels. Numbers of Ig⁺ cells were higher in the SRI than the IGI at day 12 (Fig. 1c, $p < 0.001$) and were also higher than the SRC (Fig. 1c, Fig. 4, $p < 0.001$), but numbers of Ig⁺ cells in IGI were not significantly different from IGC on day 12 (Fig. 1c, $p = 0.222$).

Infection intensity and inflammation in IGI, as determined by histology, changed over time (Fig. 1a–b, $p = 0.02$; $p < 0.001$), with both measures higher on day 12 and day 25 than on day 90. On day 25, *C. shasta* trophozoites were detected in five of nine IGI: one fish had an infection intensity score of five, the other four had a score of one. Inflammation on day 25 differed from day 12 not in severity, but in the location and distribution of host inflammatory cells (Fig. 2f–g). Host mononuclear cells were largely limited to the lamina propria and some fused villi in at least two of the fish on day 25 and trophozoites were present among the infiltrated cells. Inflammation in another two fish from this group was more diffuse, extending through the muscularis and serosal surface into the mesenteric adipose tissue. In one of these two fish, parasites were present within the adipose tissue, but numerous trophozoites were also present in the intestinal epithelium layer and destruction of villi was evident. In one fish, the steatitis extended from the serosal surface and surrounded a blood vessel. The number of Ig⁺ cells increased significantly on day 25 compared to day 12 ($p < 0.001$) in the IGI and was also significantly higher than the IGC (Fig. 1c, $p < 0.001$). By day 90, parasites were observed in only one fish and inflammation in the lamina propria was absent to minimal with lymphocytes scattered in the mesenteric adipose tissue (Fig. 2h–i). The number of Ig⁺ cells in the day 90 IGI was not significantly different from the IGC ($p < 0.462$) and the IGI Ig⁺ cells were also significantly lower at day 90 than day 25 ($p < 0.001$).

3.3 Cytokine Expression

Intestinal expression of IL-6, IL-10 and IFN γ was upregulated ($p < 0.001$) in SR fish 12 days post exposure. IFN γ was the only cytokine significantly upregulated ($p = 0.007$) in IG fish 12 days post-exposure, but expression was seven times lower than that of SR fish. Expression of IFN γ on day 25 in IG fish increased compared to day 12, but was no longer upregulated by day 90 ($p = 0.54$). TNF α was upregulated in IG fish on day 90 (Table 2, $p = 0.003$). Differences in expression were not detected for TGF β and IL-2.

4. Discussion

In this study, the responses of resistant (IG) and susceptible (SR) Chinook salmon to *C. shasta* were followed from the site of parasite invasion to the intestine. Disease was fatal in SR fish but there was clearance and resolution of the infection in IG fish through an effective inflammatory response. The relatively equal numbers of parasites penetrating the gills of IG and SR Chinook suggests that resistance to invasion by the parasite does not occur. However, as proposed in the literature [8, 9, 16, 17], resistant fish did show evidence of preventing parasite establishment in the intestine. This appears to have occurred by a twofold process; 1) limiting the number of parasites that invade/establish in the intestine, as evidenced by lower infection intensity and 2) an effective inflammatory response limiting parasite proliferation in that tissue. By 90 days resistant fish had recovered from the infection, as evidenced by elimination of parasites from the intestine coinciding with the reduction of inflammation, pro-inflammatory cytokine expression and Ig+ cells.

Differences between the timing and magnitude of the responses to parasite infection were evident between fish strains. Although both fish strains recruited Ig+ cells to the infection site, Ig+ cell recruitment occurred later in the resistant strain (day 25 as opposed to day 12 in the susceptible strain). We observed upregulation of the pro-inflammatory cytokines IFN γ and IL-6 in both fish strains, although the magnitude of expression was higher for SR than IG fish. In cold-water fish such as Chinook salmon, adaptive immunity typically occurs between six to twelve weeks post infection [31]. Because IL-6 is expressed prior to the development of an adaptive response, this cytokine may function early in the infection in a pro-inflammatory capacity [32, 33, 34]. The increase in the number of Ig+ cells in SR fish at day 12 likely represent cells secreting non-specific antibodies. Thus, increased numbers of Ig+ cells and expression of pro-inflammatory cytokines, accompanied with failure to protect the host, suggest a premature or hyperactive response in the SR fish as compared to the IG fish.

Reduced inflammation at day 12 in SR as compared to IG fish was unexpected given that cytokine expression and numbers of Ig+ cells were higher in SR fish and previous studies with susceptible fish have shown severe inflammation in moribund fish [19, 20]. Inflammation in SR on day 12 occurred only in the lamina propria and did not arrest parasite proliferation or prevent parasites from reaching the epithelium. Because fish were not sampled at a time when the fish were clinically diseased, it is unknown if inflammation, cytokine expression and numbers of Ig+ cells would have continued to increase with disease progression. Earlier sampling may also have aided in a better understanding of inflammation patterns. For example, increased expression of the pro-inflammatory cytokines that respond quickly to infection, such as TNF α and IL-1 β , may have occurred prior to day 12. If upregulation occurred prior to our sampling in IG fish, this could explain the increased histological inflammation in IG fish compared to SR fish. An alternate hypothesis for the lower inflammation observed in SR fish at day 12 is that IL-10, a potent anti-inflammatory cytokine, may have suppressed both IL-6 and IFN γ [35, 36]. In addition, although cytokine expression was upregulated in SR at day 12, this expression may not have been sufficient to elicit the extent of inflammation observed in IG Chinook.

While both Chinook strains were exposed to the same infectious dose and the number of infecting parasites was similar, the infection intensity measured in the intestine of SR on day 12 was approximately four times higher than that of IG fish. We offer two explanations for the reduced number of parasites in the intestine of the IG strain by day 12. First, parasites could be eliminated prior to arrival in the intestine (i.e. in the blood) and/or second, elimination could occur upon arrival in the intestine before our first sampling time. Although not examined in this study, we hypothesize that the immune response in resistant Chinook reduces the numbers of proliferating parasites in the blood during migration from the gills to the intestine between the time of initial infection and our first measurement on day 12. We have some support for this hypothesis; blood collected daily from IG fish after exposure to the parasite for two weeks indicated a reduction in parasite numbers by day 12 [37]. These additional defenses may limit the number of parasites invading the intestine and account for the difference in the timing of the response in the intestine between the Chinook strains.

Over the course of the infection, the numbers of parasites in the intestine of resistant fish remained low, likely because proliferation was controlled by the inflammatory response. Additionally, the high survival of infected IG fish in the presence of these extensive inflammatory lesions and resolution of the infection in cohorts by day 90 supports the successful regulation of this response. By day 90, there were no significant differences in IFN γ expression, inflammation and the number of Ig $^{+}$ cells from controls, indicating a return to basal levels. Therefore, although both strains of fish elicited an inflammatory response and recruited Ig $^{+}$ cells to the intestine, the response was only effective for IG Chinook and was likely aided by the reduction of parasites prior to entering the intestinal tissues.

As a continued inflammatory response in the intestine would lead to immunopathology, downregulation of inflammatory cytokines prevent host tissue damage [38]. Although our study was limited to a single exposure to the parasite, decreased expression of IFN γ along with increased numbers of Ig $^{+}$ cells suggests a switch from a T helper 1 (Th1) to a T helper 2 (Th2) response in resistant fish. For fish living in a river, exposure to *C. shasta* actinospores would be continuous, potentially leading to chronic inflammation. Thus, a switch to Th2 would prevent chronic inflammation and may stimulate a protective antibody response [39]. In susceptible rainbow trout infected with a presumably non-pathogenic strain of *C. shasta*, parasites were identified in the presence of increased mucosal IgT antibody [24]. Although it is not known if this response is protective against *C. shasta*, protective antibody responses have been demonstrated in fish surviving other myxozoan infections [40, 41, 42, 43].

Previous studies examined the host response to *C. shasta* in susceptible rainbow trout infected with a different parasite genotype than used in this study [22, 23]. Although this complicates comparisons between resistance strategies, we did see some similarities between infections among fish species. Ibarra et al. [8] noted formation of granulomata around the parasite and hypothesized that *C. shasta* resistance results from the ability of the fish to mount an effective immune response. Bartholomew et al. [16] found parasites in the lumen without triggering inflammation and low mortality in resistant trout, suggestive of

tolerance. In our study, although we did not observe granulomata or parasites in the lumen, resistant IG fish exhibited greater inflammation and had lower infection intensities compared to susceptible fish. Over time, infection intensities decreased, suggesting that the inflammatory response and/or the increase in numbers of Ig⁺ cells was capable of eliminating parasites prior to sporulation or movement into the intestinal lumen. Although the details of our observations were different than those observed in rainbow trout and steelhead, the hypotheses regarding resisting parasite establishment (in the intestine) as well as the development of an effective immune response in the form of an inflammatory response and/or antibody response are supported in this study.

Similarities exist in the host response against *C. shasta* and other myxozoans with tropism for the intestine of its fish host. In sharpsnout sea bream (*Diplodus puntazzo*) infected with *Enteromyxum leei*, the immune response is decreased compared with resistant gilthead sea bream (*Sparus aurata*), although infection severity is higher and disease progression is faster in the sharpsnout sea bream [44, 45]. This finding is comparable to the response of the susceptible SR strain to *C. shasta* infection on day 12 of our study, where parasite intensity was higher in the resistant IG strain, but inflammation was lower. Gilthead sea bream also demonstrated recruitment of lymphocytes to the intestine and upregulation of pro-inflammatory cytokines early in infection [45, 46]. This response is similar to the inflammatory response in the resistant IG fish in our study. Gilthead sea bream also displayed upregulation of anti-inflammatory cytokines later in the study, which may have correlated with a decrease in lymphocyte infiltration into intestinal tissues [45, 46]. This trend was not detected in our study, although a sample point between 25 and 90 days may have provided further resolution of the response. Thus, an increased immune response in the intestine appears to be a common response to invasion by myxozoan parasites with intestinal tropism, but resistant fish appear to be able to mount a more effective response than susceptible fish.

5. Conclusions

Results of this study demonstrated that fish with increased resistance to enteronecrosis elicit an effective immune response capable of eliminating parasites. The effectiveness of the response is dependent on the parasite dose, as resistant fish can succumb to disease at high doses. The trends in cytokine expression and inflammatory cell recruitment to the intestine differed in timing and magnitude between susceptible and resistant fish. Expression of IFN γ , IL-6 and IL-10 was higher in susceptible fish at day 12, but expression continued to increase in resistant fish by day 25. Despite the increased expression of cytokines later in resistant fish, the histological inflammatory response was more intense in resistant fish at day 12. This may be due to upregulation of pro-inflammatory cytokines in resistant fish earlier in the infection or the expression of IL-10, a potent anti-inflammatory in susceptible fish. We also observed differences in the numbers of parasites detected in the intestine, with fewer parasites in the intestine of resistant fish. Because resistance is a genetically controlled trait [4], these fish may elicit an earlier immune response en route or upon arrival to the intestine. One mechanism for this may be pathogen recognition receptors. To better understand the role of innate immunity in prevention of clinical disease, future studies should determine the parasite dose range in which an effective immune response can be

initiated but not overwhelmed. Additionally, new insights on the infectious dose, route of infection and timing of host response to the parasite in the intestine create new opportunities to investigate the possibility of a protective antibody response to *C. shasta*.

Acknowledgments

We thank Don Stevens, Harriet Lorz, and Jill Pridgeon for maintaining polychaetes in the lab and assistance with fish care and sampling; Gerri Buckles for help with qPCR. We thank Jerry Heidel at the Veterinary Diagnostic Laboratory at Oregon State University for his assistance with interpretation of the histopathology, Stephen Atkinson for assistance with image formatting and Chris Bayne for his comments on this manuscript. Fish were provided by Oregon Department of Fish and Wildlife's Salmon River Hatchery and California Department of Fish and Wildlife's Iron Gate Hatchery. This work was funded by Oregon Sea Grant under project number R/RCF-24 and award number NA060AR4170010 from the U.S. Department of Commerce National Oceanic and Atmospheric Administration's National Sea Grant College Program and appropriations made by the Oregon State Legislature. Funds were also provided by the National Science Foundation (Grant NSF-IOS-1022300 MCB-0719599 (to J.O.S.) and the National Institutes of Health (R01GM085207 to J.O.S. and J.B.)

References

1. Ibarra AM, Hedrick RP, Gall GAE. Inheritance of susceptibility to *Ceratomyxa shasta* (Myxozoa) in rainbow trout and the effect of length of exposure on the liability to develop ceratomyxosis. *Aquaculture*. 1992; 104:219–229.
2. Bartholomew JL, Whipple MJ, Campton D. Inheritance of resistance to *Ceratomyxa shasta* in progeny from crosses between high- and low- susceptibility strains of rainbow trout (*Oncorhynchus mykiss*). *Bull Natl Res Inst Aquac*. 2001; 5:71–75.
3. Ibarra AM, Hedrick RP, Gall GAE. Genetic analysis of rainbow trout susceptibility to the myxosporean *Ceratomyxa shasta*. *Aquaculture*. 1994; 120:239–262.
4. Nichols KM, Bartholomew JL, Thorgaard GH. Mapping multiple genetic loci associated with *Ceratomyxa shasta* resistance in *Oncorhynchus mykiss*. *Dis Aquat Org*. 2003; 56:145–154. [PubMed: 14598990]
5. Ching HL, Munday DR. Susceptibility of six Fraser River Chinook salmon stocks to *Ceratomyxa shasta* and the effects of salinity on ceratomyxosis. *Can J Zool*. 1984; 62:1081–1083.
6. Hemmingsen AR, Holt RA, Edwing RD, McIntyre JD. Susceptibility of progeny from crosses among three stocks of Coho salmon to infection by *Ceratomyxa shasta*. *Am Fish Soc*. 1986; 115:492–495.
7. Ching HL, Parker L. Experimental exposure of trout and salmon from 12 British Columbian stocks to the myxozoan parasite *Ceratomyxa shasta*. *J Aquat Anim Health*. 1989; 1:205–208.
8. Ibarra AM, Gall GAE, Hedrick RP. Susceptibility of two strains of rainbow trout *Oncorhynchus mykiss* to experimentally induced infections with the myxosporean *Ceratomyxa shasta*. *Dis Aquat Org*. 1991; 10:191–194.
9. Bartholomew JL. Host resistance to infection by the myxosporean parasite *Ceratomyxa shasta*: a review. *J Aquat Anim Health*. 1998; 10:112–120.
10. Ratliff DE. *Ceratomyxa shasta*: Longevity, distribution, timing and abundance of the infective stage in central Oregon. *Can J Fish Aquat Sci*. 1983; 40:1622–1632.
11. Foott JS, Harman R, Stone R. Effect of water temperature on non-specific immune function and ceratomyxosis in juvenile Chinook salmon and steelhead from the Klamath River. *Calif Fish Game*. 2004; 90:71–84.
12. Stocking RW, Holt RA, Foott JS, Bartholomew JL. Spatial and temporal occurrence of the salmonid parasite *Ceratomyxa shasta* in the Oregon-California Klamath River Basin. *J Aquat Anim Health*. 2006; 18:194–206.
13. Ray RA, Rossignol PA, Bartholomew JL. Mortality threshold for juvenile Chinook salmon *Oncorhynchus tshawytscha* in an epidemiological model of *Ceratomyxa shasta*. *Dis Aquat Org*. 2010; 93:63–70. [PubMed: 21290897]

14. Udey LR, Fryer JL, Pilcher KS. Relation of water temperature to ceratomyxosis in rainbow trout (*Salmo gairdneri*) and coho salmon (*Oncorhynchus kisutch*). J Fish Res Board Can. 1975; 32:1545–1551.
15. Ray RA, Holt RA, Bartholomew JL. Relationship between temperature and *Ceratomyxa shasta*-induced mortality in Klamath river salmonids. J Parasitol. 2012; 98:520–526. [PubMed: 22746389]
16. Bartholomew JL, Ray E, Torell B, Whipple MJ, Heidel JR. Monitoring *Ceratomyxa shasta* infection during a hatchery rearing cycle: comparison of molecular, serological and histological methods. Dis Aquat Org. 2004; 62:85–92. [PubMed: 15648834]
17. Bartholomew JL, Smith CE, Rohovec JS, Fryer JL. Characterization of a host response to the myxosporean parasite, *Ceratomyxa shasta* (Noble), by histology, scanning electron microscopy and immunological techniques. J Fish Dis. 1989; 12:509–522.
18. Bartholomew JL, Whipple MJ, Stevens DG, Fryer JL. The life cycle of *Ceratomyxa shasta*, a myxosporean parasite of salmonids, requires a freshwater polychaete as an alternate host. J Parasitol. 1997; 83:859–868. [PubMed: 9379291]
19. Bjork SJ, Bartholomew JL. Invasion of *Ceratomyxa shasta* (Myxozoa) and migration to the intestine with a comparison between susceptible and resistant fish hosts. Int J Parasitol. 2010; 40:1087–1095. [PubMed: 20385137]
20. Bjork SJ, Bartholomew JL. Effects of *Ceratomyxa shasta* dose on a susceptible strain of rainbow trout and comparatively resistant Chinook and coho salmon. Dis Aquat Org. 2009; 86:29–37. [PubMed: 19899347]
21. Hallett SL, Ray RA, Hurst CN, Holt RA, Buckles GR, Atkinson SD, Bartholomew JL. Density of the waterborne parasite, *Ceratomyxa shasta*, and its biological effects on salmon. App Environ Microbiol. 2012; 78:3724–3731.
22. Atkinson SD, Bartholomew JL. Disparate infection patterns of *Ceratomyxa shasta* (Myxozoa) in rainbow trout (*Oncorhynchus mykiss*) and Chinook salmon (*Oncorhynchus tshawytscha*) correlate with internal transcribed spacer-1 sequence variation in the parasite. Int J Parasitol. 2010; 40:599–604. [PubMed: 19895812]
23. Atkinson SD, Bartholomew JL. Spatial, temporal and host factors structure the *Ceratomyxa shasta* (Myxozoa) population in the Klamath River basin. Infect Gen Evol. 2010; 10:1019–1026.
24. Zhang Y-A, Salinas I, Li J, Parra D, Bjork S, Zhen X, LaPatra SE, Bartholomew J, Sunyer JO. IgT, a primitive immunoglobulin class specialized in mucosal immunity. Nature Immunol. 2010; 11:827–836. [PubMed: 20676094]
25. Hallett SL, Bartholomew JL. Application of a real-time PCR assay to detect and quantify the myxozoan parasite *Ceratomyxa shasta* in river water samples. Dis Aquat Org. 2006; 71:109–118. [PubMed: 16956058]
26. Palenzuela O, Trobridge G, Bartholomew JL. Development of a polymerase chain reaction diagnostic assay for *Ceratomyxa shasta*, a myxosporean parasite of salmonid fish. Dis Aquat Org. 1999; 36:45–51. [PubMed: 10349552]
27. Palenzuela O, Bartholomew JL. Molecular tools for the diagnosis of *Ceratomyxa shasta* (Myxozoa). Mol Diag Salm Dis. 2002:285–298.
28. Bartholomew JL, Arkoosh MR, Rohovec JS. Demonstration of the specificity of the salmonid humoral response to *Renibacterium salmoninarum* with a monoclonal antibody against salmonid immunoglobulin. Journal of Aquat Anim Health. 1991; 3:254–259.
29. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res. 2001:29–45. [PubMed: 11125041]
30. Pfaffl MW, Horgan GW, Dempfle L. Relative expression software tool (REST) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res. 2002:30–36.
31. Sitja-Bobadilla A. Fish immune response to Myxozoan parasites. Parasite. 2008; 15(3):420–425. [PubMed: 18814716]
32. Secombes, CJ.; Zou, J.; Bird, S. Fish cytokines: Discovery, activities and potential applications. In: Zacccone, G.; Meseguer, J.; Garcia-Ayala, A.; Kapoor, BG., editors. Fish Defenses Volume 1, Immunology. Science Publishers, Enfield, NH, USA; 2009. p. 1-36.

33. Kaneda M, Odaka T, Suetake H, Tahara D, Miyadai T. Teleost IL-6 promotes antibody production through STAT3 signaling via IL-6R and gp130. *Dev Comp Immunol.* 2012; 38(2):224–231. [PubMed: 22469658]
34. Chen HH, Lin HT, Fuong YF, Han-You Lin J. The bioactivity of teleost IL-6: IL-6 protein in orange spotted grouper (*Epinephalus coioides*) induced Th2 cell differentiation pathway and antibody production. *Dev Comp Immunol.* 2012; 38(2):285–294. [PubMed: 22858412]
35. Sugamata R, Suetake H, Kikuchi K, Suzuki Y. Teleost B7 expressed on monocytes regulates T cell responses. *J Immunol.* 2009; 182(11):6799–6806. [PubMed: 19454675]
36. Grayfer L, Hodgkinson JW, Hitchen SJ, Belosevic M. Characterization and functional analysis of goldfish (*Carassius auratus* L.) interleukin IL-10. *Mol Immunol.* 2011; 48(4):563–571. [PubMed: 21111485]
37. Bjork, S. Factors affecting the *Ceratomyxa shasta* infectious cycle and transmission between polychaete and salmonid hosts. Dissertation, Oregon State University; 2010. <http://hdl.handle.net/1957/15435>.
38. Sitjà-Bobadilla A, Caldúch-Giner J, Saera-Vila A, Palenzuela O, Álvarez-Pellitero P, Pérez-Sánchez J. Chronic exposure to the parasite *Enteromyxum leei* (Myxozoa: Myxosporidia) modulates the immune response and the expression of growth, redox and immune relevant genes in gilthead sea bream, *Sparus aurata* L. *Fish Shellfish Immunol.* 2008; 24:610–619. [PubMed: 18343157]
39. Jankovic D, Sher A, Yap G. Th1/Th2 effector choice in parasitic infection: decision making by committee. *Curr Opin Immunol.* 2001; 13:403–409. [PubMed: 11498295]
40. Sitjà-Bobadilla A, Palenzuela O, Ríaza A, Macías MA, Álvarez-Pellitero P. Protective acquired immunity to *Enteromyxum scopthalmi* (Myxozoa) is related to specific antibodies in *Psetta maxima* (L.) (Teleostei). *Sc and J Immunol.* 2007; 66:26–34.
41. Clifton-Hadley RS, Bucke D, Richards RH. Proliferative kidney disease of salmonid fish: a review. *J Fish Dis.* 1984; 7:363–377.
42. Klontz GW, Rourke AW, Eckblad W. The immune response during proliferative kidney disease in rainbow trout: a case history. *Vet. Immunol. Immunopathol.* 1986; 12:387–393. [PubMed: 3765361]
43. Foott JS, Hedrick RP. Seasonal occurrence of the infectious stage of proliferative kidney disease (PKD) and resistance of rainbow trout, *Salmo gairdneri* Richardson to re-infection. *J Fish Biol.* 1987; 30:477–483.
44. Muñoz P, Cuesta A, Athanassopoulou F, Golomazou H, Crespo S, Padrós F, Sitjà-Bobadilla A, Albiñana G, Esteban MA, Alvarez-Pellitero P, Meseguer J. Sharpshout sea bream (*Diplodus puntazzo*) humoral immune response against the parasite *Enteromyxum leei* (Myxozoa). *Fish Shellfish Immunol.* 2007; 23:636–645. [PubMed: 17475509]
45. Alvarez-Pellitero P, Palenzuela O, Sitjà-Bobadilla A. Histopathology and cellular response in *Enteromyxum leei* (Myxozoa) infections of *Diplodus puntazzo* (Teleostei). *Parasitol Int.* 2008; 57:110–120. [PubMed: 18373973]
46. Pérez-Cordón G, Estensoro I, Benedito-Palos L, Caldúch-Giner JA, Sitjà-Bobadilla A, Pérez-Sánchez J. Can a parasitic infection modulate the expression of interleukin genes in a fish-myxozoan system? *Fish Shellfish Immunol.* 2013; 34:1672.

Highlights

- Resistance to parasite invasion at the attachment site did not occur
- Infection intensity in the intestine early in the infection was four times higher in susceptible fish than resistant fish suggesting that immune responses en route or soon after the parasite reaches the intestine may limit infection intensity in resistant fish
- Resistant fish limited parasite proliferation in the intestine
- Cytokine expression levels and timing differed between strains, with IL-6, IL-10 and IFN γ upregulated in susceptible fish at day 12. IFN γ was the only cytokine upregulated in resistant fish, with the highest fold change at 25 days post infection.
- Resistant fish recovered from infection and resolved the inflammation

Fig. 1.

Average Infection Intensity (a), Inflammation (b) and numbers of Ig+ cells (c) for susceptible Salmon River (SR) and resistant Iron Gate (IG) Chinook salmon on days 12, 25 and 90. Each bar represents nine fish. For the top and middle figures, upper case letters indicate differences between fish strains on day 12 and lower case letters indicate differences among days for IG using Kruskal-Wallis with Wilcoxon rank sum for pairwise comparisons. All controls scored 0. For the bottom figure, upper case letters indicate significant differences between fish strain-day using a two-way ANOVA with Tukey's test for highly significant differences. Control values are not depicted for continuity.

Fig. 2.

Inflammation in Salmon River (SR) and Iron Gate (IG) Chinook salmon stained with Giemsa. Arrows indicate parasite foci. Areas of inflammation in the lamina propria of SR intestine at day 12 post infection adjacent to tissue with no inflammation (a, b). Inflammation in IG intestine in serosa, lamina propria, muscularis and stratum granulosum at day 12; although lymphocytes are numerous, no parasites are present (c–e). Inflammation in IG intestine at day 25 in lamina propria, surrounding blood vessels and resulting distortion of villi (f, g). Inflammation in IG intestine at day 90 largely limited to the lamina propria; no parasites are present (h, i).

Fig. 3.

Ceratomyxa shasta parasite foci (indicated with arrows) in Salmon River (SR) and Iron Gate (IG) Chinook salmon stained with Giemsa. Trophozoites in the intestinal epithelium of SR in the absence of inflammation at day 12 (a, b). Parasites within foci of inflammation in the intestinal serosa of IG at day 12 (c, d).

Fig. 4.

Ig+ cells (brown) in the intestinal tissue of control (a) and infected (b) Salmon River Chinook salmon on day 12. Sections were counterstained with hematoxylin.

Table 1

Primer sequences, primer concentration, reaction efficiency, product size, and GenBank accession number for Chinook salmon cytokine expression analysis.

Gene	Primer sequences (5'-3')	Primer concentration and efficiency	Product size (bp)	GenBank #
β actin	(F) GGACTTTGAGCAGGAGATGG	4 μ M, 99.4%	186	Chinook salmon
	(R) ATGATGGAGTTGTAGGTGGTCT			FJ546418
IL-1 β	(F) ACCGAGTTCAAGGACAAGGA	6 μ M, 99.9%	181	Chinook salmon
	(R) CATTTCATCAGGACCCAGCAC			DQ778946
IL-2	(F) TTTCCTTTTGACGCTTTTCTCA	4 μ M, 99.6%	204	Rainbow trout
	(R) CGAGGCATTCTACTTTACAGT			NM_001164065
IL-6	(F) CAGTTTGTGGAGGAGTTTCAGA	2 μ M, 99.3%	118	Rainbow trout
	(R) TGTGTAGTTTGAGGTGGAGCA			NM_001124657
IL-10	(F) CTACGAGGCTAATGACGAGC	6 μ M, 99.5%	100	Rainbow trout
	(R) GATGCTGTCCATAGCGTGAC			AB118099
IFN γ	(F) CAACATAGACAACTGAAAGTCCA	4 μ M, 99.4%	129	Chinook salmon
	(R) ACATCCAGAACCACACTCATCA			GT897806
TGF β	(F) AGATAAATCGGAGAGTTGCTGTG	2 μ M, 99.9%	275	Rainbow trout
	(R) CCTGCTCCACCTTGTTGT			X99303
TNF α	(F) ACCAAGAGCCAAGAGTTGAAC	2 μ M, 98.0%	154	Chinook salmon
	(R) CCACACAGCCTCCATAGCCA			DQ778945

Table 2

Fold change in intestinal expression of cytokine genes of susceptible Salmon River (SR) and resistant Iron Gate (IG) strains of Chinook salmon after infection with *Ceratomyxa shasta*. Numbers in bold indicate significant changes in treatment from control ($p < 0.05$).

Cytokine	SR-day 12		IG-day 12		IG-day 25		IG-day90	
	Fold Change	SE	Fold Change	SE	Fold Change	SE	Fold Change	SE
TNF α	1.04	-2.67-2.51	-1.01	-3.79-3.41	1.59	-1.57-3.40	2.26	1.05-4.48
IL-1 β	-1.44	-4.05-2.15	-1.22	-3.28-3.43	-1.20	-3.53-3.11	1.03	-1.67-1.77
IL-6	5.51	2.27-13.27	2.55	1.02-7.39	4.59	-1.70-111.63	-1.18	-2.11-1.51
IL-10	14.22	7.22-39.46	3.55	-3.45-47.03	2.29	-4.69-24.17	-1.09	-1.95-1.56
IFN γ	28.97	13.01-66.55	3.65	1.32-11.67	5.43	-1.16-41.23	-1.19	-2.78-1.85