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Title: What Makes a Fish Resistant? Comparative Genomics and Transcriptomics of *Oncorhynchus mykiss* with Differential Resistance to the Parasite *Ceratonova shasta*

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Jerri L. Bartholomew

The myxozoan *Ceratonova shasta* is an intestinal parasite of salmon and trout that causes ceratomyxosis, a disease characterized by severe inflammation of the intestine that can lead to hemorrhaging, necrosis, and death of the fish host. The parasite is endemic to the Pacific Northwest of the United States and Canada, where it has been linked to the decline of wild fish stocks. The parasite exerts a strong selective force on its fish host, and fish populations from *C. shasta* endemic watersheds become genetically fixed for resistance to ceratomyxosis. This contrasts with fish from watersheds where the parasite is not established, who are highly susceptible the disease, with a single spore capable of causing a lethal infection. Management of the disease relies on selective stocking of resistant fish, however, even these fish can succumb to the infection. Understanding the genetic and immunological basis of resistance to this disease would provide the framework for the development of therapeutics and identification of genetic markers that could be used in selective breeding. In this project, we employed a comparative transcriptomics and genomics approach to understand how resistant and susceptible strains of *Oncorhynchus mykiss* (rainbow trout/steelhead) respond to *C. shasta* infection and identify the genomic loci conferring resistance. We found that infection by *C. shasta* has an immunosuppressive effect in both resistant and susceptible fish, with IFNγ and interferon stimulated genes being downregulated in the gills, which is the portal of

entry for the parasite. Once the parasite reaches the intestine, resistant fish quickly respond with an IFNγ driven T_H1 response, and upregulation of MHC class I genes and genes involved in antigen processing and presentation. This response suggests that *C. shasta* has an intracellular phase during its migration to the intestine, which may represent a form of immune evasion that causes the host to initiate a cytotoxic T-cell response that is ineffective against the extracellular stages in the intestine. Susceptible fish, on the other hand, had no detectable immune response to the parasite reaching the intestine, highlighting the importance of parasite recognition in the different infection outcomes of these fish. In addition to a more rapid immune response, resistant fish have a tissue level response that limits the spread of the parasite within the intestine and allows them to regenerate their intestinal lining. Additionally, we have identified a region on chromosome Omy9 that significantly affects the odds of surviving *C. shasta* infection, and a region on Omy11 with a lesser contribution to survival. Taken together, this research represents a major step forward in our understanding of both the genetic and immunological basis of resistance to this important parasite.

What Makes a Fish Resistant? Comparative Genomics and Transcriptomics of *Oncorhynchus mykiss* with Differential Resistance to the Parasite *Ceratonova shasta*

by
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I understand that my dissertation will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my dissertation to any reader upon request.

Damien E. Barrett, Author

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CONTRIBUTION OF AUTHORS

For the second manuscript, Dr. Itziar Estensoro processed tissue samples and interpreted the histology and Dr. Ariadna Sitjà-Bobadilla helped interpret the histology and provided valuable feedback on the manuscript.

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CHAPTER 1: INTRODUCTION

Introduction to myxozoa

Myxozoa is diverse group of microscopic obligate endoparasites with over 2,400 known species distributed among fresh and saltwater habitats worldwide (Zhang, 2013). Belonging to phylum Cnidaria (which includes jellyfish, hydras, sea anemones, and corals), they possess nematocysts (also known as a cnidocyte or polar capsule) which are specialized stinging organelles that are a defining characteristic of Cnidarians (Holland et al., 2011; Shpirer et al., 2014). Unlike their free-living ancestors, myxozoans are highly reduced in terms of size, tissue complexity and genome size (Chang et al., 2015; Okamura and Gruhl, 2016). Myxozoans utilize their nematocysts as a means of anchoring to their hosts and initiating the infection process. A defining trait of myxozoans is their two-host, two-spore lifecycle which alternates between a vertebrate host (usually fish) and an invertebrate host (usually annelid) (Fig. 1.1). The two spore stages are the actinospore, which infects the fish host, and the myxospore which infects the annelid host. Infections in the fish host are generally asymptomatic, however some myxozoans are known to cause significant disease or death (Sitjà-Bobadilla, 2008).

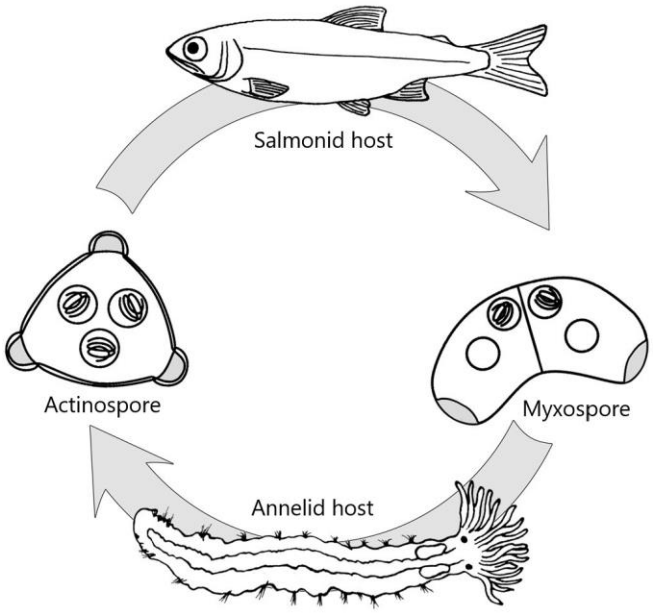

Figure 1.1. The lifecycle of *Ceratonova shasta*, a myxozoan that alternates between a salmonid fish host and the annelid Manayunkia occidentalis. Image credit: S. Atkinson.

The myxozoan parasite Ceratonova shasta

Ceratonova shasta (formerly *Ceratomyxa shasta*) is an intestinal parasite of salmonid fish that causes ceratomyxosis, a disease characterized by inflammation, hemorrhaging and necrosis of the intestine and ultimately death of the fish host. *C. shasta* was first described by Noble 1950 after an unknown parasite decimated the rainbow trout being reared at Crystal Lake Hatchery at Mount Shasta, California (Noble, 1950). Since that time, *C. shasta* has negatively affected numerous salmon and trout populations throughout the Pacific Northwest of the United States and Canada, where it is enzootic. *C. shasta* is established in all of the major river systems of this region, including the Columbia, Sacramento, Klamath, Rogue, and Fraser rivers (Bartholomew, 1998; Hoffmaster et al., 1988). Natural infections of *C. shasta* are known to occur in most, if not all, salmonids species present within the region and fish may become infected as juveniles or adults (Stinson et al., 2018). Infected fish are unable to transmit the infection to other fish, as *C. shasta* requires the annelid *Manayunkia occidentalis* to complete its lifecycle and generate fish-infecting actinospores (Figure 1.1) (Atkinson et al., 2020; Bartholomew et al., 1997). Infection of the fish host is initiated when the actinospore attaches to the gill epithelium and migrates into the blood vessels. Secondary cells are then produced which either multiply within the blood vessels or migrate to the lower intestine (Bjork and Bartholomew, 2010). In the intestine, the secondary cells migrate through the tissue layers to the mucosal epithelium and begin sporogenesis, which results in myxospores being released into the intestinal lumen (Yamamoto and Sanders, 1979).

Factors affecting the outcome of infection

Not all infections result in ceratomyxosis, as the outcome of the infection depends on both biotic (infectious dose, parasite virulence, innate host resistance) and abiotic (temperature, water velocity and flow rate) factors. Water temperature is perhaps the most important abiotic factor as it affects both the development rate and longevity of the parasite as well as the physiology of the host (Foott et al., 2007). Natural infections are typically observed at water temperatures between 7 and 25 C with increasing water temperature linked to higher mortality and decreased time to death (Ray et al., 2012; Udey et al., 1975). Water velocity and flow are also important, effecting both the number of actinospores that come in contact with the gill surface and the transmission efficiency (Hallett and Bartholomew, 2008; Bjork and Bartholomew, 2009b; Hallett et al., 2012).

A major breakthrough in our understanding of *C. shasta* infection dynamics came with the discovery that the parasite exists as complex of host-specific genotypes. The three identified genetically distinct strains (genotypes 0, I, and II) are associated with different salmonid hosts and varying degrees of parasite virulence (Alama-Bermejo et al., 2020; Atkinson and Bartholomew, 2010a; Atkinson et al., 2018). Genotype 0 is the least virulent, causing chronic infections in rainbow trout/steelhead (*Oncorhynchus mykiss*) that are subclinical with low parasite proliferation (Atkinson and Bartholomew, 2010b; Stinson et al., 2018). Genotype I and II can both be highly virulent and are able to cause the disease symptoms that are classically associated with ceratomyxosis (Bjork and Bartholomew, 2010; Bjork et al., 2014). Genotype I is specific to Chinook salmon (*O. tshawytscha*), while genotype II has a wider host range, opportunistically infecting numerous salmonids, including rainbow trout/steelhead, coho salmon (*O. kisutch*), sockeye/kokanee (*O. nerka*), chum salmon (*O. keta*), pink salmon (*O. gorbuscha*) and cutthroat trout (*O. clarkii*). Infections of non-native salmonids have also been reported in brown trout (*Salmo trutta*), brook trout (*Salvelinus fontinalis*), and Atlantic salmon (*Salmo salar*) (Stinson and Bartholomew, 2012; Stinson et al., 2018). Genotype II can be further subdivided into biotypes IIR and IIC, which both cause disease in allopatric rainbow trout, but only IIC is known to cause significant disease in coho salmon (Hurst et al., 2012). For the remainder of this chapter, all mentions of *C. shasta* will refer to the virulent genotypes I and II, unless noted otherwise.

A critical factor in the development of disease is the innate resistance of the host. *C. shasta* is not established in all river systems and allopatric salmonid populations are highly susceptible to the parasite, with a single actinospore capable of causing a lethal infection (Zinn et al., 1977; Buchanan et al., 1983; Ratliff, 1983; Bjork and Bartholomew, 2009a). This strongly contrasts with sympatric salmonid populations, which have evolved a high degree of resistance to the parasite and typically require exposure to thousands of spores before disease or mortality occurs (Ray et al., 2010). A series of inheritance studies conducted on sympatric and allopatric fish populations have demonstrated that the resistance trait is fixed within a population and shows little variation (Bartholomew et al., 2001; Buchanan et al., 1983; Hemmingsen et al., 1986; Ibarra et al., 1992, 1994; Nichols et al., 2003). When high and low susceptibility fish are crossed, the offspring have a mortality threshold intermediate that of the parents. This indicates that the alleles conferring resistance are dominant, and that being homozygous at these loci

confers greater resistance to *C. shasta*. Nichols et al. conducted the first genetic mapping experiment that attempted to identify the genetic loci associated with *C. shasta* resistance in *O. mykiss.* Although limited by the small number of fish in their analysis, the authors found multiple genomic regions associated with resistance, confirming earlier work that indicated the trait was polygenic (Ibarra et al., 1994; Nichols et al., 2003). However, these studies lacked both sequencing data and a reference genome, and so were unable to identify specific loci or candidate genes for the trait.

Progression of the infection in the fish host

While the route of parasite invasion and migration is the same in both resistant and susceptible fish hosts (Bjork and Bartholomew, 2010), some differences in how the infection progresses have been observed between the two.

Beginning with susceptible fish; once the parasite reaches the lower intestine, it migrates through the serosa and lamina propria and begins to proliferate within the mucosal epithelia. This triggers a host inflammatory response, consisting mainly of infiltrating lymphocytes and monocytes, with some neutrophils and macrophages being noted (Bartholomew et al., 1989; Bjork et al., 2014). The lamina propria becomes enlarged due to the inflammatory response and the intestinal epithelia becomes necrotic and begins to slough off. The parasite remains in the circulatory system for the duration of the infection, and after proliferation occurs in the lower intestine, it is often seen invading the liver, pyloric caeca, and kidneys (Bjork and Bartholomew, 2010). As the disease progresses, the lower intestine becomes grossly enlarged, bloody, and necrotic and overt clinical signs of ceratomyxosis appear, including darkening of the skin pigmentation, lethargy, anorexia, and a bloody and swollen vent. Additionally, ascites may collect in the coelom, causing severe abdominal bloating (Schafer, 1968; Wales and Wolf, 1955). Susceptible fish are unable to mount an effective immune response to *C. shasta*, and infection with a compatible genotype appears to always result in mortality.

In resistant fish, the outcome of infection falls on a continuum that is heavily influenced by temperature and the number of infecting parasites per unit time. When the parasite dose is low, fish are able to prevent the parasite from invading the intestine and no pathological changes are observed (Bartholomew et al., 1989). Moderate doses are associated with intestinal inflammation, few clinical disease signs, and little to no mortality. Bartholomew et al. (Bartholomew et al., 2004) examined the intestines of steelhead and cutthroat trout chronically

exposed to *C. shasta* at low ambient water temperatures (< 10° C) and found infections characterized by foci of granulomatous inflammation and large numbers of parasites on the mucosal surface. However, mortality rates were low, and several fish had signs of fibrosis in their intestine, indicating they were recovering from the infection. Other studies involving sub-lethal exposures of steelhead and Chinook have noted containment of the parasite within well-defined granulomas (Foott and Stone, 2004; Foott et al., 2007; Ibarra et al., 1992). The infectious dose required to produce mortality in resistant stocks is not well characterized and likely differs between species and stocks. For resistant Iron Gate Chinook salmon, Ray et al. estimated a non-linear mortality threshold of $7.7 \pm 2.1 \times 10^4$ actinospores fish^{-1} when exposed in the Klamath River (Ray et al., 2010). When this mortality threshold is passed, the disease appears to progress the same as in susceptible fish (Bartholomew et al., 1989; Bjork and Bartholomew, 2010), although bloating due to ascites has not been reported.

Investigations into the mechanism of resistance

These observations lead to, and supported, a hypothesis of resistance put forth by Ibarra et al. (Ibarra et al., 1994), in which resistance depends on two interacting mechanisms: 1) control of parasite invasion or establishment and 2) the ability to mount an effective immune response against parasites that succeeded in invading the intestine. Exclusion of the parasite at the site of entry was initially proposed (Bartholomew et al., 1989), but comparison of resistant and susceptible fish exposed to the same dose of *C. shasta* found no evidence for this, with equivalent parasite burdens in the gills of both phenotypes (Bjork and Bartholomew, 2010; Bjork et al., 2014). This agrees with earlier work by Ibarra et al. that examined the response of resistant and susceptible rainbow trout with experimentally induced *C. shasta* infections that bypassed the natural route of parasite entry by injecting *C. shasta* into the abdomen (Ibarra et al., 1991). The authors found that mortality occurred in dose-dependent manner, with the lowest dose causing 13% mortality in the resistant fish and 90% mortality in the susceptible fish. These observations strongly suggest that resistance occurs beyond the gills, either in the bloodstream as the parasite migrates to the intestine, or when the parasite initially reaches the intestine.

Overview of the teleost immune system

The immune system of teleost fish is very similar to that of higher vertebrates, both having innate and adaptive immune systems with many of the same molecules and cells at their disposal (Uribe et al., 2018). The primary difference between fish and mammals lies in the

poikilotherm nature of fish, with the ambient water temperature affecting many physiological processes, including the immune system (Quinn et al., 2017). The innate immune system (sometimes referred to as nonspecific immunity) is comprised of physical barriers to infection (skin, mucus, gills), antimicrobial compounds (lysozyme, complement, proteases), and innate immune cells (macrophages, dendritic cells, granulocytes) that can recognize and destroy certain pathogens. Pathogens that overcome the innate defenses of their host then encounter the adaptive immune system (sometimes referred to as specific immunity), which involves the generation of T and B cells (T and B lymphocytes) that can recognize specific antigens present on, or released by, the pathogen. Cytotoxic T-lymphocytes can directly recognize and kill infected host cells, while T-helper cells orchestrate the overall immune response by activating or repressing certain cells or pathways depending on the effector cell type they differentiate into (T_H1, T_H2, T_H17, T_{REG}) (Ashfaq et al., 2019). B cells produce antibodies (immunoglobulins, Ig) that can recognize and bind to a specific pathogen or a toxin they produce. Fish possess three immunoglobulin isotypes, which have specialized roles in systemic (IgM, IgD) and mucosal (IgT) immunity (Mashoof and Criscitiello, 2016).

Which of these systems are engaged by a pathogen is highly variable and depends on the type of pathogen (virus, bacteria, parasite), the infection site (organ, tissue type, intracellular or extracellular), and whether or not the host has germline encoded receptors capable of recognizing the pathogen. The pathogen may also modulate the hosts immune response to its own benefit, something commonly observed in parasitic infections (Bhavsar et al., 2007; Cecílio et al., 2014; Maizels and McSorley, 2016; Freyberg and Harvill, 2017). Several reviews have been published on the fish immune response to parasite infections (Alvarez-Pellitero, 2008; Jones, 2001; Piazzon et al., 2013) and myxozoans specifically (Gómez et al., 2014; Sitjà-Bobadilla, 2008). The remainder of this chapter will focus on what is currently known about the immune response to *C. shasta*, while providing an overview of what is known about other fish-myxozoan systems.

Studies on the antibody response to C. shasta

The first insights into the immunological basis of resistance came in 2010, when researchers discovered that *C. shasta* in the intestine of rainbow trout were coated in IgT, a previously uncharacterized immunoglobulin isotype specialized in mucosal immunity (Zhang et al., 2010). The authors found parasite-specific titers of IgM in the serum and IgT in the intestinal mucus three months after infection. It should be noted that these were susceptible rainbow trout

naturally exposed in the river to unknown genotype(s), although infection with genotype 0 can be inferred from the river location and the lack of mortality in these fish. This finding lead to the work of Taggart-Murphy et al. (Taggart-Murphy, 2018), which examined the role IgT in susceptible rainbow trout infected with *C. shasta.* They initially found that IgM and IgT were upregulated in both chronic genotype 0 infections and acute high mortality genotype IIR infections. The authors suggested that the failure of this adaptive immune response to prevent mortality was due to it 1) being insufficient to clear the parasite, 2) occurring too late, after the tissue has been damage and/or 3) contributing to the observed host damage and pathological changes. A follow up experiment examined the effect of depleting IgT^+ B cells in susceptible rainbow trout prior to infecting them with genotype II. Their initial hypothesis was that fish depleted of IgT^+ B cells would have higher pathogen loads, as the parasite would be able to proliferate unimpeded. Unexpectedly, the IgT-depleted fish had lower pathogen loads than their respective controls, suggesting that IgT does not play a role in resistance to *C. shasta*. However, as the authors noted, depletion of IgT^+ B cells may have affected other immune parameters, such as expression of IL-10, which would have an indirect effect on pathogen loads by affecting inflammation levels. It is important to highlight that all of these studies were conducted on susceptible rainbow trout, and that role of IgT and other immunoglobulins has not been investigated in *C. shasta* infections of resistant fish.

Studies on the cytokine response during C. shasta infections

Cytokines are small secreted proteins involved in the communication and interactions between cells. They function in a wide array of physiological processes but are most commonly known for their role in the immune system, where they help orchestrate the immune response to pathogens or other insults (Zhang and An, 2007; Turner et al., 2014). Key cytokines are often interrogated during an infection to infer the wider immune responses at play.

The first study of this kind for *C. shasta* was done by Bjork et al., comparing the expression levels of five cytokines (TNFα, IL-1β, IL-6, Il-10, IFNγ) in resistant and susceptible Chinook infected with genotype I (Bjork et al., 2014). The resistant Chinook eventually cleared the infection and had elevated levels of IFNγ at 12- and 25-days post infection (dpi), and TNFα at 90-dpi. Susceptible fish showed elevated expression of IL-6, IL-10, and IFNγ at 12-dpi, but succumbed to the infection before the subsequent sampling period. The authors noted that despite the higher expression of the pro-inflammatory IL-6 and IFNγ in susceptible fish at 12-

dpi, histological examination revealed more extensive inflammation in the intestines of resistant fish. They suggested that this may be due to earlier upregulation of pro-inflammatory cytokines in resistant fish, or the expression of the anti-inflammatory IL-10 in susceptible fish.

Hurst et al. later examined the expression of seven cytokines (TNFα, IL-1β, IL-6, IL-8, IL-10, IFNγ, TGFβ) in the intestine and spleen of susceptible Chinook infected with either genotype I or II, or a combination of both genotypes (Hurst et al., 2019). They found no difference in the expression of TNFα, TGFβ, or IL-1β in any of the treatments, whereas the expression of IL-6, IL-8, IL-10, and IFNγ varied among treatments and tissues. Genotype I infections were characterized by increased immune gene expression in the spleen, indicating a systemic response to infection. Genotype II infections were characterized by increased immune gene expression the intestine, indicating a localized immune response. Infection with both genotypes produced cytokine profiles similar to genotype I infections in the spleen and genotype II infections in the intestine. These results indicate that different genotypes may elicit different immune responses. However, it is unclear if this is driven by host or parasite factors and if this differential response also occurs in resistant Chinook.

Recent work by Taggart-Murphy et al. (Taggart-Murphy, 2018) measured the expression of IL-6, IL-8, IL-10, and IFNγ in the intestine of allopatric rainbow trout infected with either genotype 0 or IIR. IFNγ and IL-10 were upregulated at all timepoints in both infections, with stronger induction in genotype IIR infections. IL-6 expression was not significantly different at any point in either infection, and IL-8 was mildly induced in genotype IIR infections. The lack of pro-inflammatory cytokine expression in genotype 0 is consistent with the chronic, non-destructive nature of these infections. The early and dramatic increase in IL-10 expression in genotype IIR infections supports a hypothesis by Bjork et al. (Bjork et al., 2014) that early induction of IL-10 in susceptible fish may interfere with the effectiveness of their inflammatory response.

Caution must be taken in interpreting the results of these studies, as all three of them utilized qPCR primers specific to single gene. All salmonid species are the product of a relatively recent whole genome duplication event (Berthelot et al., 2014). As a result, their genomes contain a high number of paralogous genes, which may or may not have retained identical functions. Even when paralogs retain the same function, they often diverge in their regulation, showing temporal- or tissue-specific expression (Allendorf and Thorgaard, 1984). For the

cytokines in question, multiple copies have been found in salmonids to date (Secombes et al., 2011; Zou and Secombes, 2016). With that in mind, these studies overall suggest a prominent role of IFNγ in the immune response to *C. shasta*, having been induced in different species and stocks of fish, and in infections with all three genotypes. They also indicate that IL-10 may be inhibiting the inflammatory response of susceptible fish, leading to reduced parasite clearance, a well-documented phenomenon in other infectious diseases (Couper et al., 2008).

The immune response in other fish-myxozoan systems

Whirling disease, caused by *Myxobolus cerebralis*, is perhaps the most well-known and well-studied myxozoan disease (Gilbert and Granath, 2003). Like *C. shasta*, *M. cerebralis* is able to infect most salmonid species (Sollid et al., 2003), but is primarily known for the devastating effect it has had on wild and managed trout populations throughout the United States and Europe (Hedrick et al., 1998; Bartholomew and Reno, 2002). Rainbow trout are the most susceptible species to *M. cerebralis* infection, and much of what we know about the disease comes from controlled laboratory experiments with them (Markiw, 1989, 1991, 1992). The parasite initially infects the fish-host through the gills or epidermis and then migrates through the nervous tissue to the cartilage of the brain and spinal cord, where sporogenesis occurs (El-Matbouli et al., 1995). There are varying levels of resistance to whirling disease among salmonid species and high and low-susceptibility strains of rainbow trout have been the subject of several in-depth immunological studies (Baerwald, 2013; Baerwald et al., 2008, 2011; Fetherman et al., 2012; Severin and El-Matbouli, 2007). Despite this, the reasons for these differences in susceptibility and mechanism(s) that drive resistance remain largely unknown. However, there are some strong similarities to what is observed in *C. shasta* infections. Firstly, fish that are susceptible to *M. cerebralis* appear unable to mount and effective immune response and suffer high mortality rates. Secondly, resistant and susceptible fish have a strong IFNγ driven T cell response, however, the response in susceptible fish appears delayed and excessive, possibly contributing to the observed tissue damage (Saleh et al., 2019).

Proliferative kidney disease (PKD), caused by the malacosporean *Tetracapsuloides bryosalmonae* (Canning et al., 1999, 1999), is another high impact myxozoan disease of farmed and wild salmonids throughout the United States and Europe (Hedrick et al., 1993; Okamura et al., 2011). Similar to *M. cerebralis*, infection occurs through the gills or epidermis and disease is primarily reported in trout. PKD is a chronic disease characterized by granulomatous-like lesions

and lymphocytic hyperplasia of the interstitial kidney tissue alongside excessive immunoglobulin production (Gorgoglione et al., 2013; Hedrick et al., 1993). Temperature plays a critical factor in the development of disease, with few clinical signs and low mortality occurring at temperatures below 15° C (Okamura et al., 2011). How the fish-host responds to infection is also temperature-dependent. At 12° C they exhibit a predominant T_H1 response with upregulation of IFNγ, while the response at 15° C is characteristic of a T_H2 response with upregulation of IL-4/13 (Bailey et al., 2017). Fish that recover from the infection develop a strong immunity to reinfection, possibly due to the production of parasite-specific IgM (Bailey et al., 2017).

Kudoa thyrsites is a marine parasite infecting over 35 species of fish worldwide, including many salmonids (Whipps and Kent, 2006). The parasite is intracellular, infecting the myofibrils of the somatic muscle. While these infections are not associated with host mortality, they do lead to the formation of cysts in the muscle tissue that can induce postmortem myoliquefaction (soft flesh syndrome) through the release of proteolytic enzymes (Dawson-Coates et al., 2003). This poses a major economic threat to many commercial fisheries due to loss of fillet quality (Moran et al., 1999). Very little is known about the host response to infection, outside of two studies done on Atlantic salmon. Jones et al. (Jones et al., 2016) demonstrated that Atlantic salmon that recover from infection with *K. thyrsites* develop a strong resistance to reinfection (Jones et al., 2016). Braden et al. later found that this acquired resistance was associated with a population of $MHII^+$ cells that surrounded and infiltrated infected muscle cells. Many of these cells were shown to be $MHII^+/CD83^+$ dendritic cells and some were observed to have engulfed myxospores. IgM+ and CD8α+ cells were also found near the site of infection, alongside upregulation of IgT, IgM, IL-12, T cell receptor, NK-lysin, and CD8α, which is suggestive of a T_H1 cytotoxic T cell response (Braden et al., 2017). Although the authors did not assess IFNγ levels, its induction can be inferred from the upregulation of IL-12 which is part of the IL-12/IFNγ pathway that leads to T cell activation (Rosenzweig and Holland, 2005).

Enteromyxum scopthalmi is the causative agent of enteromyxosis, a highly pernicious disease of turbot (*Scophthalmus maximus* L.) with mortality rates usually reaching 100% (Branson et al., 1999; Quiroga et al., 2006). Unlike other myxozoan diseases discussed thus far, *E. scopthalmi* can be transmitted fish-to-fish via contaminated stool from diseased fish. This leads to severe outbreaks of the disease in turbot aquaculture, with no available treatment outside

of culling the infected tanks (Sitjà-Bobadilla and Palenzuela, 2012). The parasite infects the digestive tract, and after a long pre-patent period, clinical signs of the disease develop: anemia, anorexia, intestinal lesions, catarrhal enteritis, and leukocyte depletion (Ronza et al., 2019). An exacerbated local inflammatory response is believed to play a role in the formation of intestinal lesions, with an increase in inducible nitric oxide synthase and TNFα expression found in the gut of infected turbot (Losada et al., 2012; Ronza et al., 2015a, 2015b). Turbot that survive *E. scopthalmi* infection develop an acquired protection that is associated with the production of parasite-specific antibodies (Sitjà-Bobadilla, 2004). However, most studies indicate that humoral immunity is delayed and ineffective against the parasite (Bermúdez et al., 2006; Sitjà-Bobadilla et al., 2006).

An RNA-seq analysis of turbot with advanced infections found more evidence for an overactive inflammatory response, with upregulation of pro-inflammatory genes and downregulation of antioxidant defense genes (Robledo et al., 2014). The same study found downregulation of numerous genes involved in adaptive immunity, antigen presentation, T_h17 cells, and interferon production. These observations lead the authors to suggest there was breakdown in the components bridging the innate and adaptive responses in these fish. A follow up RNA-seq study examined the response of turbot during the early stages of the infection, when clinical signs were mild (Ronza et al., 2016). Evidence of a possible intracellular phase was found, with activation of the RIG-I-like receptor and IFNγ pathways, which are both involved in the immune response to intracellular pathogens. This aligns with prior histology work that reported a potential intracellular phase for the parasite during the early stages of the infection (Palenzuela et al., 2002; Redondo et al., 2004). Both RNA-seq studies found evidence of pathogen recognition, with upregulation of CD209 and other C-type lectins, which supports previous work on the involvement of lectins in recognizing *E. scopthalmi* (Redondo and Alvarez-Pellitero, 2010; Redondo et al., 2008).

Enteromyxum leei is a closely related myxozoan that also causes enteromyxosis. While *E. scopthalmi* primarily infects turbot, *E. leei* is able to infect a wide range of fish species with varying degrees of host susceptibility. Sharpsnout sea bream (*Diplodus puntazzo*) and tiger puffer (*Takifugu rubripes*) are highly susceptible to the parasite, while infection of gilthead sea bream (*Sparus aurata*) causes subchronic disease with mortality rates below 20% (Sitjà-Bobadilla and Palenzuela, 2012). These differences in pathogenesis have been partially attributed

to the ability of gilthead sea bream to maintain their intestinal epithelial integrity, even when heavily infected (Fleurance et al., 2008; Ronza et al., 2020). The immune response of gilthead sea bream to *E. leei* infection appears to be a T cell response primarily, with recruitment of lymphocytes from the spleen and head kidney to the intestine (Estensoro et al., 2014). The cytokine profiles indicate a mixed population of T_H1, T_H17, and cytotoxic T cells at the infection site (Piazzon et al., 2018). In a study by Davey et al., gilthead sea bream were chronically exposed to *E. leei* for 113 days, classified as infected or uninfected and samples were analyzed using a microarray (Davey et al., 2011). Uninfected fish had elevated expression of interferon stimulated genes and MHC class II genes involved in antigen processing and presentation, whereas fish that became infected exhibited a marked downregulation of immune and acute phase response genes.

While these studies involved a diverse set of myxozoans and fish hosts, certain throughlines are present. Rather than falling on a continuum, resistance to a myxozoan presents as an almost binary trait with fish populations being either highly resistant or highly susceptible. When fish are susceptible, their immune response appears largely ineffective, often being delayed and excessively inflammatory, whereas resistant fish seem to engage with the parasite sooner and with a more effective inflammatory response. A conserved role for IFNγ in the immune response to myxozoans is apparent; its upregulation can either be directly found or inferred in most studies (summarized in Table 1.1). What role immunoglobulins play is less clear cut. Excessive production contributes the pathological changes of PKD, while at the same time acquired immunity to both *T. bryosalmonae* and *K. thyrsites* appears to involve IgM. Both turbot and rainbow trout make parasite-specific antibodies, although they appear ineffective in reducing disease, at least in susceptible fish. Future studies should emphasize comparisons between resistant and susceptible fish, as trying to determine what may be going wrong in susceptible fish is difficult without knowing what's going right in resistant fish.

Table 1.1 Expression of interferon gamma (IFNγ) during myxozoan infections. Asterisks (*) denote studies in which the cytokine was not directly measured, but the regulation of which can be inferred from other measurements and observations.

Species	Phenotyp	Pathogen	IFNγ	Source
Chinook	Resistant	*C. shasta*	Upregulated	Bjork et al. 2014
Chinook	Susceptibl	*C. shasta*	Upregulated	Bjork et al. 2014
Chinook	Susceptibl	*C. shasta*	Upregulated	Hurst et al. 2019
Rainbow Trout	Susceptibl	*C. shasta*	Upregulated	Taggart-Murphy
Gilthead Sea	Resistant	*E. leei*	Upregulated*	Davey et al. 2011
Gilthead Sea	Susceptibl	*E. leei*	Downregulated	Davey et al. 2011
Gilthead Sea	Unknown	*E. leei*	Upregulated	Piazzon et al. 2019
Turbot	Susceptibl	*E. scopthalmi*	Downregulated	Robledo et al. 2014
Turbot	Susceptibl	*E. scopthalmi*	Upregulated	Ronza et al. 2016
Atlantic Salmon	Resistant	*K. thyrsites*	Upregulated*	Braden et al. 2017
Rainbow Trout	Resistant	*M. cerebralis*	Upregulated	Baerwald et al. 2008
Rainbow Trout	Susceptibl	*M. cerebralis*	Upregulated	Baerwald et al. 2008
Rainbow Trout	Resistant	*M. cerebralis*	Upregulated	Baerwald et al. 2013
Rainbow Trout	Susceptibl	*M. cerebralis*	Upregulated	Baerwald et al. 2013
Rainbow Trout	Susceptibl	*T.*	Upregulated	Gorgoglione et al.
Brown Trout	Tolerant	*T.*	Upregulated	Bailey et al. 2019

Research objective

Despite the widespread threat that myxozoans pose to both wild and managed fish populations, there remains no viable prophylactic or therapeutic treatment for the diseases they cause. Large knowledge gaps also remain in what is known about the host-myxozoan dialogue and what drives resistance, both of which are critical for developing treatments and management strategies. The *O. mykiss/C. shasta* system offers a tractable model for studying the immune response to myxozoans and the genetic basis of host resistance. Rainbow trout and steelhead are among the most widely cultivated fish species in the world and serve as a model species for many areas of research. Because of this, a large knowledge base exists for the species and they are the only Pacific salmonid with a fully sequenced genome. Within the Pacific Northwest, there exists many natural populations of *O. mykiss* on the phenotypic extremes of resistance. This avoids the time-consuming process of creating resistant and susceptible fish lines for research. Additionally, *C. shasta* is one of the few myxozoans whose life cycle is both known and maintained in a laboratory setting, permitting controlled laboratory challenges.

The goal of this dissertation is to understand how resistant and susceptible *O. mykiss* respond to *C. shasta* infection and to identify the immunological and genetic basis of resistance. This objective is addressed in the following chapters:

- **Chapter 2** is a comparative transcriptomics study of the early response to *C. shasta* infection in both resistant and susceptible steelhead. I hypothesize that early recognition of the parasite is critical for resistance and that susceptible fish would initially fail to recognize and respond to the infection.
- **Chapter 3** examines the transcriptomic response of resistant steelhead with severe *C. shasta* infections. Much of what we know about the host response to *C. shasta*, and other myxozoans, comes from studies of highly susceptible fish. Understanding how resistant fish respond to infection provides a valuable point of comparison for these studies that helps explain the differences in susceptibility.
- **Chapter 4** is a genetic mapping study that seeks to identify the genomic regions that confer resistance to *C. shasta.* A backcross generation of rainbow trout were created and phenotyped. RAD-seq was then used to identify loci associated with resistance.

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CHAPTER 2: A TALE OF TWO FISH: COMPARATIVE TRANSCRIPTOMICS OF RESISTANT AND SUSCEPTIBLE STEELHEAD FOLLOWING EXPOSURE TO *CERATONOVA SHASTA* HIGHLIGHTS DIFFERENCES IN PARASITE RECOGNITION

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ABSTRACT

Diseases caused by myxozoan parasites represent a significant threat to the health of salmonids in both the wild and aquaculture setting, and there are no effective therapeutants for their control. The myxozoan *Ceratonova shasta* is an intestinal parasite of salmonids that causes severe enteronecrosis and mortality. Most fish populations appear genetically fixed as resistant or susceptible to the parasite, offering an attractive model system for studying the immune response to myxozoans. We hypothesized that early recognition of the parasite is a critical factor driving resistance and that susceptible fish would have a delayed immune response. RNA-seq was used to identify genes that were differentially expressed in the gills and intestine during the early stages of *C. shasta* infection in both resistant and susceptible steelhead (*Oncorhynchus mykiss*). This revealed a downregulation of genes involved in the IFN-γ signaling pathway in the gills of both phenotypes. Despite this, resistant fish quickly contained the infection and several immune genes, including two innate immune receptors were upregulated. Susceptible fish, on the other hand, failed to control parasite proliferation and had no discernible immune response to the parasite, including a near-complete lack of differential gene expression in the intestine. Further sequencing of intestinal samples from susceptible fish during the middle and late stages of infection showed a vigorous yet ineffective immune response driven by IFN-γ, and massive differential expression of genes involved in cell adhesion and the extracellular matrix, which coincided with the breakdown of the intestinal structure. Our results suggest that the parasite may be suppressing the host's immune system during the initial invasion, and that susceptible fish are unable to recognize the parasite invading the intestine or mount an effective immune response. These findings improve our understanding of myxozoan-host interactions while providing a set of putative resistance markers for future studies.

INTRODUCTION

Ceratonova shasta (syn. *Ceratomyxa shasta*) is a myxozoan parasite of salmonid fish that is endemic to most river systems in the Pacific Northwest of the United States (Bartholomew et al., 1997; Stinson et al., 2018). It is recognized as an economically important pathogen of both wild and hatchery-reared salmonids (Bartholomew et al., 2004; Ratliff D. E., 1981; Stinson and Bartholomew, 2012; True et al., 2016) and has been linked to population-level declines (Fujiwara et al., 2011; Hallett et al., 2012). *C. shasta* has a broad host range and is able to infect

most, if not all, native salmonid species (Stinson et al., 2018). The initial site of infection is the gills, where the parasite spore attaches to the epithelium prior to invading the blood vessels and beginning replication. Travelling via the bloodstream, it reaches the intestine 4 to 5 days after the initial infection, where it continues to replicate and undergoes sporogenesis (Bjork and Bartholomew, 2010). Severe infections result in enteronecrosis (ceratomyxosis) and death of the host. Fish stocks in the Pacific Northwest are highly divergent in their innate resistance to *C. shasta* induced mortality: those originating from *C. shasta* endemic watersheds exhibit a high degree of resistance (Hallett et al., 2012; Ray et al., 2010), whereas allopatric fish are highly susceptible (Bjork and Bartholomew, 2009a; Hallett et al., 2012). Numerous studies have demonstrated that resistance to *C. shasta* is a genetically controlled trait that shows little variation within a given population (Buchanan et al., 1983; Hemmingsen et al., 1986; Ibarra et al., 1992, 1994; Bartholomew et al., 2001; Nichols et al., 2003).

While the innate resistance of the host is a primary factor in the outcome of infection, disease severity falls on a spectrum that is heavily influenced by the exposure dynamics, which include exposure concentration and duration, water temperature, and parasite virulence (Hallett et al., 2012). At the very low end of this spectrum, susceptible fish appear unable to mount an effective immune response to *C. shasta* and suffer mortality rates at or near 100% at doses as low as one spore per fish (Bjork and Bartholomew, 2009a; Ratliff, 1983). When resistant fish are exposed under similar conditions, few if any parasites reach the intestine and no clinical signs of disease are observed (Bartholomew et al., 1989; Foott and Stone, 2004; Hurst et al., 2014). However, if the exposure dose is high, typically greater than 10,000 spores, resistant fish may succumb to the infection and the disease progresses as it does in susceptible fish (Bjork and Bartholomew, 2010; Ibarra et al., 1991). When resistant fish experience more intermediate exposure conditions, *C. shasta* is observed reaching the intestine but the fish are able to control and eventually clear the infection (Bjork et al., 2014). Bartholomew et al. found that resistant steelhead (*Oncorhynchus mykiss*) and cutthroat trout (*O. clarkii*) chronically exposed to *C. shasta* at low temperatures (< 10° C) had infections characterized by large numbers of parasites on the intestinal mucosal surface and multiple foci of inflammation in that tissue (Bartholomew et al., 2004). However, sporogenesis was not observed, mortality rates were low, and observations of fibrosis in histological sections suggested that fish were recovering from the infection. Containment of the parasite in well-defined granulomas has also been observed in sub-

lethal exposures of resistant steelhead trout and Chinook salmon (*O. tshawytscha*) (Foott and Stone, 2004; Foott et al., 2007; Ibarra et al., 1991).

Understanding the host response to infection is complicated by the fact that *C. shasta* is a species complex, comprised of four distinct genotypes that have different salmonid host associations: genotype 0 with *O. mykiss*; genotype I with Chinook salmon; and genotype II, which is considered a generalist that is able to infect multiple fish species but contains a mix of two genetically distinct subtypes named after their associated hosts: IIR for rainbow trout (freshwater strain of *O. mykiss)* and IIC for coho salmon (*O. kisutch*) (Stinson et al., 2018; Atkinson and Bartholomew, 2010a, 2010b; Alama-Bermejo et al., 2019). Along with different host specificities, these genotypes have different effects on their hosts. Genotype 0 typically causes chronic infections with no apparent morbidity or mortality. In contrast, genotypes I and II may be highly pathogenic in their respective hosts, causing the disease signs that are classically associated with *C. shasta* infections.

Knowledge of the infecting genotypes, and establishment of parasite's lifecycle in a laboratory setting (Bjork and Bartholomew, 2009b), has permitted investigations of the immune response to *C. shasta* be conducted in a controlled setting with known genotypes. One of the first, by Bjork et al., compared the host response of susceptible and resistant Chinook salmon to *C. shasta* genotype I infection (Bjork et al., 2014). No difference in parasite burden at the gills was detected. However, in the intestine, resistant fish had both a lower infection intensity and a greater inflammatory response than susceptible fish and were able to eventually clear the infection. Both phenotypes had elevated expression of the pro-inflammatory cytokine IFN-γ in the intestine, but only susceptible fish had elevated levels of the anti-inflammatory cytokine IL-10. A similar trend was found in a subsequent study of susceptible rainbow trout infected with genotype IIR, with significant upregulation of IFN-γ, IL-10, and IL-6 (Taggart-Murphy, 2018). It has also been demonstrated that fish exposed to *C. shasta* are able to produce parasite-specific IgM and IgT (Hurst et al., 2019; Zhang et al., 2010). Both IgM and IgT were found to be upregulated in high mortality genotype IIR infections (Taggart-Murphy, 2018), but whether this antibody response offers any protection against *C. shasta* pathogenesis remains to be determined.

Currently, no prophylactic or therapeutic treatments exist for *C. shasta* induced enteronecrosis and efforts to manage the disease revolve around selective stocking of resistant fish. However, even resistant fish may succumb to infection (Hallett et al., 2012) and assessing

the resistance level of a fish stock requires a series of lethal parasite challenges with large groups of fish. Insight into the molecular and genetic basis of resistance will help facilitate the development of vaccines and therapeutics for this pathogen as well as provide a non-lethal biomarker for assessing a stock's resistance. More broadly, the immune response to myxozoan pathogens remains largely uncharacterized, having been explored in a limited number of species. As a result, there is a near complete lack of therapeutics or other disease control measures, an issue that is becoming more evident as aquaculture continues to increase worldwide (Sitjà-Bobadilla, 2008; Zhao et al., 2019). *C. shasta* genotype II presents a unique model for studying the immune response to myxozoans as it is highly virulent and fish hosts are either highly resistant, or completely susceptible to the parasite, rather than falling on a continuum. Additionally, the resistance phenotype of many fish stocks is already known, which avoids the issue of *ad hoc* determination of phenotype or the need to create resistance and susceptible lines of fish for research. *C. shasta* is also one of the few myxozoans whose complete life cycle is both known and maintained in a laboratory setting. The fact that *O. mykiss* is the primary fish host is also advantageous, as rainbow trout is one of the most widely studied and cultivated fish species and an extensive knowledge base exists for it, including a fully sequenced genome. Taken together, we believe that the *C. shasta-O. mykiss* system offers a tractable model for studying the immune response to myxozoans and what genes drive resistance.

With this in mind, we chose to use resistant and susceptible steelhead as model for understanding how and when the host responds to infection at the transcriptomic level. We hypothesized that early recognition of the parasite by the host was a critical factor in resistance and that susceptible fish would fail to recognize the initial infection, responding only after the parasite began to proliferate within the intestine. Conversely, we hypothesized that resistant fish would quickly recognize and respond to the infection, preventing parasite establishment in the intestine and proliferation once there. To test this, we held both phenotypes in the same tank and exposed them in parallel to *C. shasta* to ensure equivalent exposure conditions. Infected tissue was collected from both phenotypes at 1, 7, 14, and 21 days post exposure (dpe) to assess parasite proliferation using qPCR (all timepoints) and the local host immune response during the early stages of infection (1 and 7 dpe) using RNA-Seq.

MATERIAL AND METHODS

Fish

Resistant steelhead from the Round Butte Hatchery and susceptible steelhead from the Alsea Hatchery, both located in Oregon, USA, were used in this study. From each hatchery, 6 adults were collected (3 male, 3 female) and bred to create pure-parental offspring. The offspring were raised at the Oregon State University (OSU) John L. Fryer Aquatic Animal Health Laboratory in Corvallis, Oregon, USA. The fish were fed daily with a commercial diet (Bio-Oregon, Longview, Washington, USA), and reared in tanks supplied with 13.5° C specific-pathogen free (SPF) well water. Two weeks prior to the parasite challenge, the fish were fin-clipped for identification and transferred to 100-liter tanks and acclimated to 18°C. This temperature was chosen as it reflective of the river water temperatures that out-migrating salmon experience when they are exposed to *C. shasta*, and aligns with previous studies (Chiaramonte et al., 2016).

Parasite challenge

C. shasta genotype IIR actinospores were collected from two colonies of *Manayunkia occidentalis*, the freshwater annelid host (Atkinson et al., 2020), which were maintained in indoor mesocosms receiving flow-through UV-treated river water. Influent water to each colony was shut off 24 hours prior to the challenge to allow actinospores to accumulate in the mesocosm water. To ensure that both the resistant and susceptible fish were exposed to the same concentration of actinospores, 50 fish (susceptible average 42.2 ± 3.2 g; resistant average 39.4 ± 2.9 g) from each stock (differentiated on the presence of a fin clip) were placed together in identical control and treatment tanks containing 375-liters of water maintained at 18°C. Three liters of mesocosm water, which contained an estimated 4,500 actinospores based on monitoring of parasite production by qPCR (Hallett and Bartholomew, 2006), was added to the treatment tank. At the same time, three liters of water from an uninfected annelid mesocosm was added to the control tank. Fish were held on static water with aeration for 24 hours, at which time each treatment group (resistant exposed, resistant control, susceptible exposed, susceptible control) was sorted and placed into triplicate 25-liter tanks (12 total) that were randomly assigned and supplied with 18°C water. Water samples were collected from the exposure tanks immediately after the mesocosm water was added and after the fish were removed to quantify the number of *C. shasta* spores present at the beginning and end of the challenge. The water samples were

immediately filtered and prepared for qPCR following a previously described method (Hallett and Bartholomew, 2006).

Sample collection

Fish were sampled at 1, 7, 14, and 21 days post exposure (dpe), with 1 dpe corresponding to 24 hours after initiation of parasite exposure. Fish were sampled at the same time of day to minimize possible changes in gene expression due to circadian rhythms (Reebs, 2002). At each timepoint, 3 fish from each tank were euthanized with an overdose of MS-222 (tricaine methanosulfonate, Argent Laboratories, Redmond, WA, USA) for a total of 12 fish per treatment group, and 48 per timepoint. From 2 of the 3 fish, gills (1 dpe) or intestine (7, 14, 21 dpe) were collected whole and immediately placed in RNAlater and stored at 4° C for 24 hours, prior to being placed at -80° C for long term storage. From the remaining fish, gills and intestine were collected and placed in Dietrich's fixative for histology. All methods involving live fish were approved by Oregon State University's IACUC (protocol # 4660). A summary diagram of the experimental setup in shown in Figure 2.1.

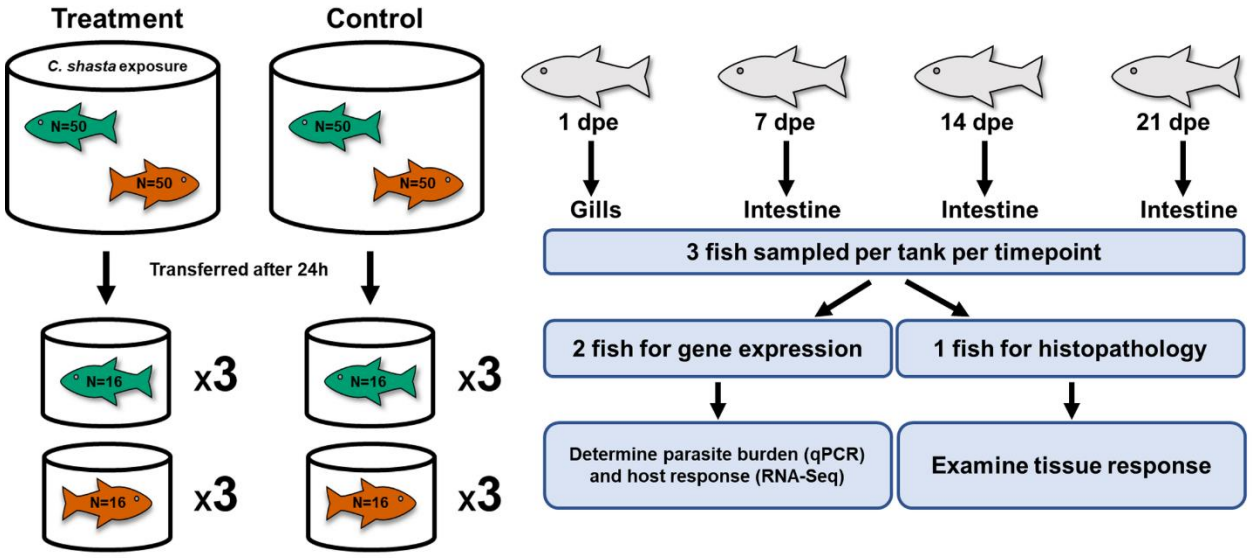

Figure 2.1. Experimental diagram of the exposure conditions and subsequent sampling of steelhead. Susceptible steelhead (green) and resistant steelhead (orange) were exposed to *Ceratonova shasta* for 24 hours and then each phenotype was separated and placed into triplicate tanks. Resistant fish had been previously fin-clipped as a means of identification. dpe = days post exposure.

Sample processing

Due to variation in the size of the gills and intestine between fish, each tissue was homogenized in liquid nitrogen using a porcelain mortar & pestle and subsampled. RNA was extracted from 25 mg of homogenized tissue using the RNeasy Mini Kit (Qiagen, catalog number 74104) following the manufacture's protocol. DNA was extracted from 25 mg of homogenized tissue from each sample using the DNeasy Blood & Tissue Kit (Qiagen, catalog number 69506) and eluted in 30 µl of Buffer AE, applied to the spin column twice, to achieve a higher concentration. The purity and concentration of the extracted RNA and DNA was assessed using a NanoDrop ND-1000 UV-Vis Spectrophotometer.

To assess the parasite load in each of the tissues, a previously developed *C. shasta* qPCR assay (Hallett and Bartholomew, 2006) was used to quantify the amount of parasite DNA present. 100 ng of DNA extracted from each sample was assayed in triplicate wells through 40 cycles using an Applied Biosystems StepOnePlus Real-Time PCR System. A sample was considered positive for *C. shasta* if all wells fluoresced and the sample was rerun if the Cq standard deviation between wells was greater than 1. On each qPCR plate, a positive control, a negative control (molecular grade water), and a standard curve of dilutions equivalent to 1, 10, 100, and 1000 actinospores was included.

Histological sections were prepared by the OSU Veterinary Diagnostic Laboratory, Corvallis, OR, USA and stained with H&E.

Sequencing

To understand the transcriptomic response of both resistant and susceptible fish during the early stages of *C. shasta* infection, mRNA from the gills at 1 dpe and from the intestine at 7 dpe was chosen for sequencing. To control for possible confounding variables, such as tank effects, six samples from each treatment group were chosen at random and were evenly split across the three tanks housing each group. 48 samples (24 per timepoint) were submitted to the Center for Genome Research and Biocomputing at OSU for library preparation and sequencing. The integrity of the RNA was confirmed by running each sample on an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). 1 µg of RNA was used for library preparation using the Illumina TruSeq™ Stranded mRNA LT Sample PrepKit according to the manufacturer's instructions (Cat. No. RS-122-2101, Illumina Inc. San Diego, CA, USA). Library quality was checked with a 4200 TapeStation System (Agilent Technologies, USA) and quantified via qPCR.

All libraries were sequenced on 4 lanes of an Illumina HiSeq 3000 as 100-bp single-end runs. The libraries were randomly distributed across the 4 lanes, 12 per lane.

Examination of the sequencing data from 7 dpe led us to sequence intestinal mRNA from susceptible fish at 14 and 21 dpe to follow the response in a progressive infection. Since we anticipated large differences in gene expression at these timepoints due to the intense histological changes observed, we chose to sequence six samples from each timepoint (3 exposed, 3 control) and do so at a higher depth of coverage to account for a greater proportion of the sequenced reads coming from parasite mRNA. 12 samples (6 per timepoint) were submitted for library preparation and sequencing as described above and were sequenced on two 100-bp single-end lanes. Resistant fish were not sequenced at these timepoints due to the low infection prevalence and intensity, the minimal transcriptomic response at 7 dpe, and because no tissue response was observed by histology.

Data analysis

Adapter sequences were trimmed from the raw reads using BBDuk (January 25, 2018 release), which is part of the BBTools package (https://jgi.doe.gov/data-and-tools/bbtools/), and all reads less than 30-bp after trimming were discarded. Library quality was assessed before and after trimming using FastQC (v 0.11.8). Reads were then mapped to the latest rainbow trout reference genome (GenBank: MSJN00000000.1) using HiSat2 (v 2.1.0) (Kim et al., 2015). Due to the high number of homeologs present in the *O. mykiss* genome (Berthelot et al., 2014), the aligned reads were filtered and sorted using SAMtools (v 1.9) (Li et al., 2009) to exclude all reads that mapped to more than one location in the genome. The number of reads that mapped to each gene was calculated using HTSeq-count (v 0.11.1) (Anders et al., 2015) and the raw counts imported in R 3.4.1 (https://www.r-project.org/) and loaded into the package DESeq2 (v 1.18.1) (Love et al., 2014). To identify potential outliers, heatmaps and PCA plots were constructed from the raw counts that were regularized log-transformed using the DESeq2 function rlogTransformation().

Differentially expressed genes (DEGs) were identified using the negative binomial Wald test in DESeq2 and were considered significant at a Benjamini–Hochberg False Discovery Rate (FDR) adjusted p-value < 0.05 and an absolute $\log_2$(fold change) > 1. Annotation of the DEGs and gene ontology (GO) enrichment was conducted with Blast2GO (v 5.2.5) (Conesa et al., 2005) with a blast e-value cutoff of $1e^{-5}$. To obtain high quality and informative annotations,

genes were preferentially annotated with the SWISS-PROT database (Bairoch and Apweiler, 2000) followed by the NCBI nonredundant database and a taxonomy filter of 'Actinopterygii' and 'Vertebrata' was applied. All genes detected within a tissue were used as the background for GO enrichment. Enriched GO terms along with their FDR-adjusted p-values, were imported into Cytoscope (v 3.7.2) (Shannon et al., 2003) for visualization with the ClueGo (v 2.5.6) (Bindea et al., 2009) plugin, which clusters genes and GO terms into functionally related networks. *O. mykiss* was chosen as the organism for Ontologies/Pathways and the GO Term Fusion option was used to merge GO terms based on similar associated genes. Volcano plots were constructed with the R package EnhancedVolcano (v 1.0.1) (Blighe et al., 2018).

RNA-seq validation by quantitative reverse transcription PCR (RT-qPCR)

The expression of four immune genes (*IFN-γ, TNF-α, IL-10, IL-1β*) found to be differentially expressed by RNA-seq were validated by quantitative reverse transcription PCR (RT-qPCR). RNA was extracted from each sample using the RNeasy Mini Kit (Qiagen) with optional on-column DNase I digestion. The purity and concentration of the extracted RNA was analyzed using a NanoDrop ND-1000 UV-Vis Spectrophotometer. 1 µg of RNA from each sample was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufactures protocol. RT-qPCR was conducted in a 96-well plate format using the Applied Biosystems StepOnePlus Real-Time PCR System. All samples were run in triplicate and each 10 µl reaction contained 2 µL of cDNA (40-fold diluted), 5 µL of 2x Power SYBR™ Green PCR Master Mix (ThermoFisher Scientific), 1 µL each of forward and reverse primers, and 1 µL molecular grade water (Lonza). Each primer pair was tested using a 5-point serial dilution to ensure an efficiency between 90-100% and melt-curve analysis was performed after each run to check for the presence of a single PCR product. The $2^{-\Delta\Delta Ct}$ method was used to determine relative gene expression with elongation factor-1α (EF-1α) serving as the housekeeping gene for normalization (Schmittgen and Livak, 2008). The list of primers used, and their amplification efficiencies are listed in S1 Table.

RESULTS

Infection of resistant and susceptible fish stocks

The exposure dose for treatment and control groups (calculated by qPCR) was 7.9×10^3 and 0 actinospores respectively (extrapolated from 1 actinospore standard). Water samples taken

at 24 hours were negative, indicating that the spores present successfully attached to the fish. Susceptible fish exposed to *C. shasta* exhibited their first clinical sign of infection at 12 dpe when they stopped responding to feed. At 21 dpe, their intestines were grossly enlarged, inflamed, and bloody, with mature *C. shasta* myxospores visible in swabs of the posterior intestine. Histology revealed a progressive breakdown of the intestinal structure in these fish (Fig. 2.2A,C). By 14 dpe, chronic inflammation could be observed throughout the intestinal submucosa (Fig. 2.2B) and by 21 dpe, all tissue layers were inflamed and sloughing of necrotic mucosal tissue was evident (Fig. 2.2C). These pathological changes were present in all three fish sampled for histology at each timepoint. No physiological changes were observed by histology in resistant fish (Fig. 2D,E).

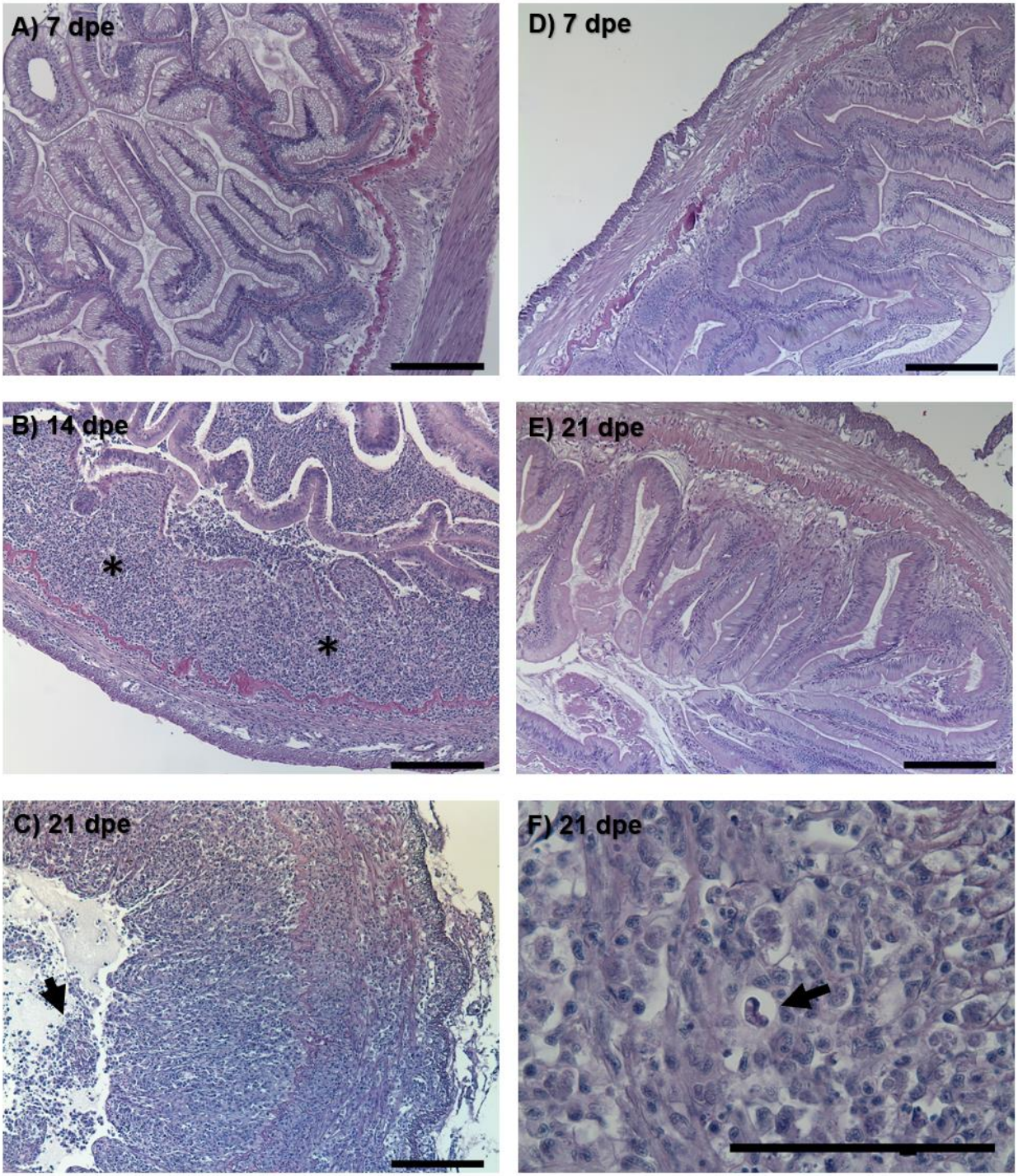

Figure 2.2. Histological sections of resistant and susceptible steelhead intestine after exposure to *Ceratonova shasta*. Susceptible fish intestine at (A) 7 days post exposure (dpe), (B) 14 dpe showing chronic inflammation (asterisks) throughout the submucosa, and (C) 21 dpe with inflammation present in all tissue layers and sloughing of necrotic epithelia (arrow). Resistant fish intestine at (D) 7 dpe and (E) 21 dpe. Mature *C. shasta* myxospore (arrow) in the intestine of susceptible fish at 21 dpe (F). All slides are stained with H&E. Bars = 100 µm.

qPCR quantification of parasite burden

C. shasta was not detected by qPCR in the gills at 1 dpe in either the resistant or susceptible fish but was detected in the intestine at 7 dpe in both phenotypes. The infection prevalence among resistant fish remained low throughout the sampling period, with less than half the fish at any timepoint having detectable levels of *C. shasta* in their intestine, and the Cq values of those fish also remained low (31.6 ± 2.2). In contrast, all susceptible fish tested from 7 dpe onwards were positive and had exponentially increasing parasite loads, with Cq values increasing from 24.8 ± 0.8 at 7 dpe to 12.6 ± 0.8 at 21 dpe (Fig. 2.3). No control fish or exposed resistant fish exhibited clinical signs of infection, and randomly selected control fish were negative by qPCR.

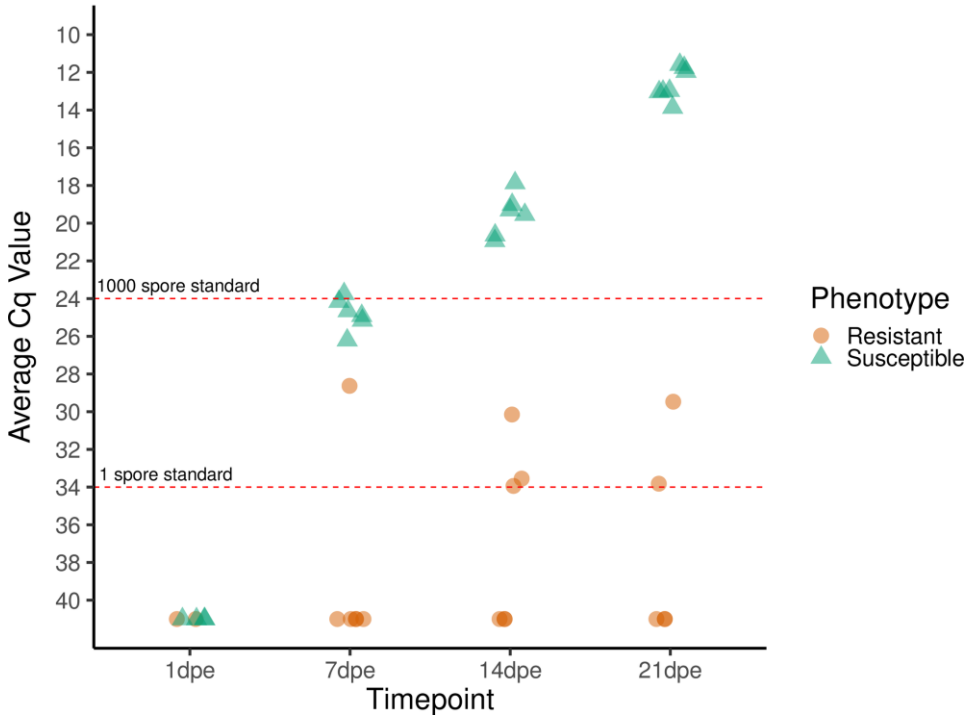

Figure 2.3. Relative quantity of *Ceratonova shasta* DNA present in the gills (1 dpe) and intestine (7, 14, and 21 dpe) of infected steelhead (*Oncorhynchus mykiss*). Each symbol represents the average quantitative cycle (Cq) of 100 ng of DNA extracted from the whole tissue (gills or intestine) of one fish that was assayed in triplicate by qPCR. Six fish of each phenotype were assayed at each timepoint. Fish that tested negative were assigned a nominal Cq value of 41. Dashed red lines indicate the average Cq values obtained from 1 and 1000 actinospore standards.

Sequencing

A total of 1.55×10^9 reads were generated from the sequencing of samples from resistant and susceptible fish at 1 and 7 dpe, with an average of 3.22×10^7 (SD ± 4.04×10^6) reads per sample (Table 2.1). 87.6% of reads could be mapped to the rainbow trout reference genome and 74.8% could be uniquely mapped to specific loci.

Table 2.1. Summary of sequencing results from gill (1 dpe) and intestine (7 dpe) of both resistant and susceptible fish.

Sequenced Reads	
Total	1,545,135,474
Removed	518,329 (0.000335%)
Mapped	1,354,217,365 (87.6%)
Uniquely Mapped	1,156,186,486 (74.8%)
Average reads per sample	32,190,322

7.80×10^8 reads were generated during the sequencing of samples from susceptible fish at 14 and 21 dpe, with an average of 6.33×10^7 (SD ± 6.00×10^6) reads per sample. The number of reads from exposed susceptible fish that could be mapped to the reference genome decreased to 83.2% at 14 dpe and 42.0% at 21 dpe, reflecting an increase in the amount of parasite RNA present (Table 2.2).

Table 2.2. Percentage of sequencing reads that mapped to the reference genome at each timepoint.

% of reads mapped				
	1 dpe	**7 dpe**	**14 dpe**	**21 dpe**
Susceptible - Exposed	87.5	88.0	83.2	42.0
Resistant - Exposed	87.7	87.4	-	-
Susceptible - Control	87.3	87.8	87.2	87.9
Resistant - Control	87.9	87.7	-	-

Gills 1 dpe - resistant and susceptible fish - differential gene expression and GO enrichment

The expression of 39,571 genes was detected from sequenced gill transcripts. DEGs responding to *C. shasta* infection were identified by comparing exposed resistant and susceptible fish to their respective controls. This identified 463 DEGs in susceptible fish and 244 in resistant fish, 66 of which were differentially expressed in both phenotypes (Fig. 2.4).

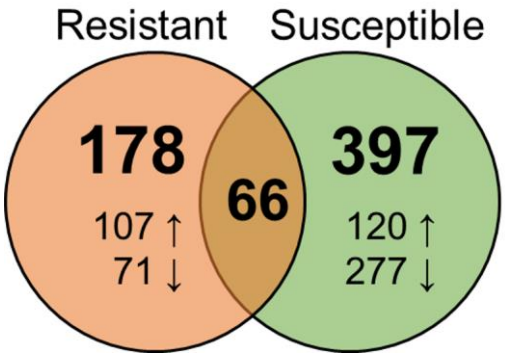

Figure 2.4. Venn Diagram showing the number of genes differentially expressed in response to *Ceratonova shasta* infection in the gills of resistant and susceptible steelhead at 1 day post exposure. Arrows indicate upregulation vs downregulation.

GO enrichment was conducted to gain insight into the biological processes, molecular functions, and cellular location of the DEGs. In susceptible fish, no specific enrichment was found among the upregulated genes and two GO terms were over-represented among genes upregulated in resistant fish (*carbon dioxide transport* and *one-carbon compound transport*). Among the downregulated genes, resistant fish had 156 enriched GO terms, and susceptible fish had 51. ClueGo analysis revealed that genes involved in the innate immune response, interferon-gamma mediated signaling pathway, response to cytokine, and response to biotic stimulus were over-represented among the downregulated genes for both resistant and susceptible fish (Figure 2.5). Many of the downregulated immune genes were shared by both phenotypes (Table 2.3), including interferon gamma 2, Interferon-induced protein 44, and several C-C motif chemokines.

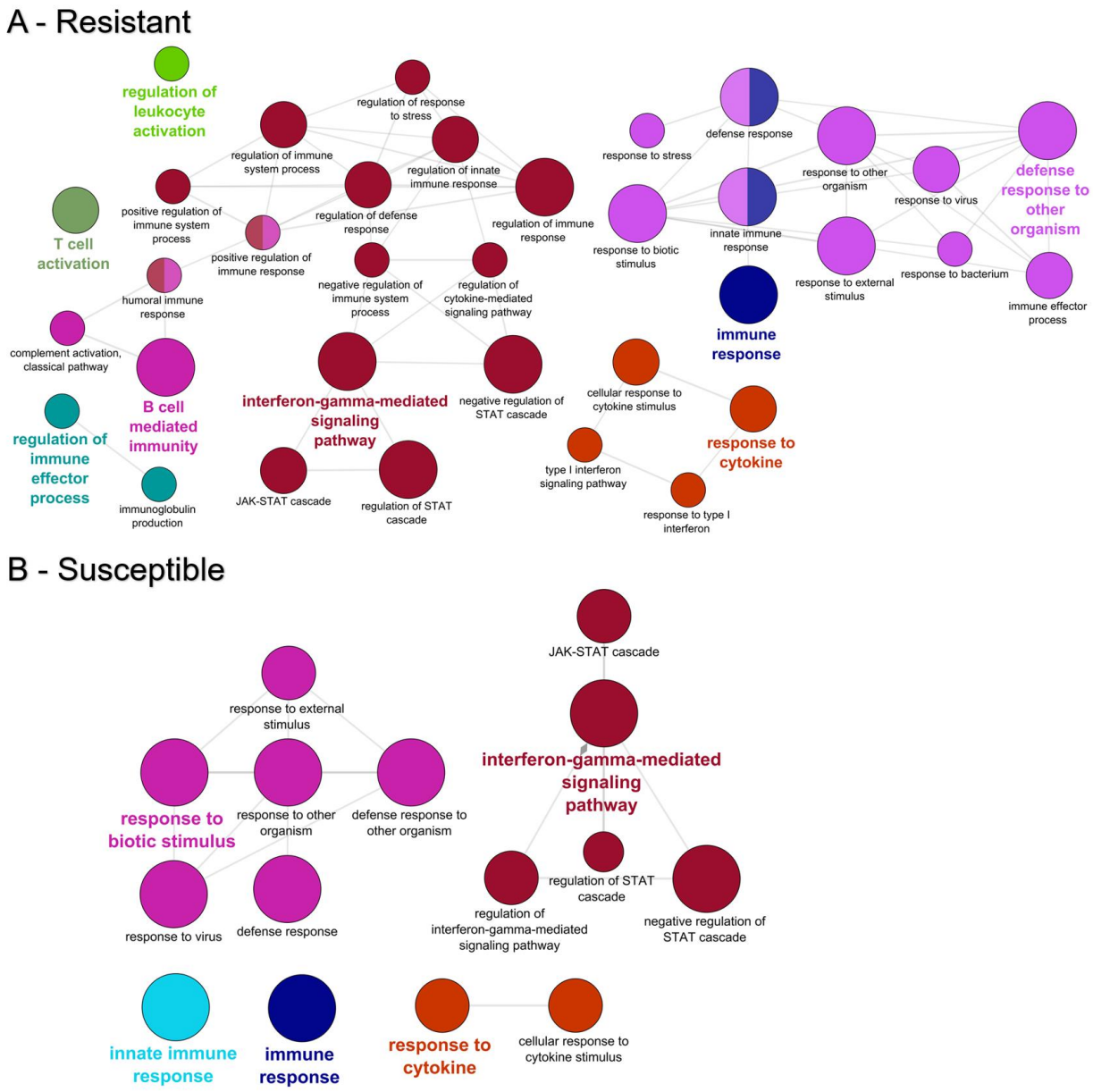

Figure 2.5. GO enrichment among the genes downregulated in the gills of resistant (A) and susceptible (B) steelhead at 1 day post exposure to *Ceratonova shasta*. Enriched gene ontology (GO) terms were grouped into functionally related nodes using the Cytoscope plugin ClueGO. Nodes are colored and grouped according to a related function and labelled by the most significant term of the group. Node size corresponds to the FDR-adjusted p-value of each GO term and is specific to each graph

Table 2.3. Select immune genes that were differentially expressed in the gills of resistant and susceptible steelhead at 1 day post exposure to *Ceratonova shasta*. Non-significant differences in expression are marked as "-".

Entrez Gene	Protein Product	Log_2-FC	Log_2-FC
ifngamma2	interferon gamma 2 precursor	-2.4	-2.5
LOC110502724	interferon-induced protein 44-like	-2.9	-2.7
LOC110491862	tumor necrosis factor receptor superfamily	-2.1	-1.8
LOC110525651	OX-2 membrane glycoprotein-like	-2.3	-1.5
LOC110509876	C-C motif chemokine 19	-2.0	-1.8
LOC110536450	C-C motif chemokine 4-like	-3.1	-2.2
LOC110514657	C-C motif chemokine 13-like	-2.5	-1.7
LOC110514021	CD83 antigen-like (1)	-2.3	-1.6
LOC110534699	CD83 antigen-like (2)	-1.3	-1.5
cxcf1b	chemokine CXCF1b precursor	-1.7	-1.3
socs1	suppressor of cytokine signaling 1	-1.8	-1.3
LOC110488345	antigen peptide transporter 2-like	-1.0	-1.0
LOC110536401	interleukin-1 beta-like	-1.0	-
irf-1	interferon regulatory factor 1	-1.8	-
il17c1	interleukin 17C1 precursor	-	-1.6
LOC110497745	interleukin-17F-like	-	-2.5
LOC110520644	interferon-induced GTP-binding protein Mx-	-	-2.2
LOC110502724	interferon regulatory factor 1-like	-	-2.9
cxcl13	chemokine CXCL13 precursor	-	-4.6
LOC110535225	B-cell receptor CD22-like	-	1.2
LOC110534952	CD209 antigen-like protein E	-	1.1
LOC110487421	NOD-like receptor C5	1.7	-
LOC110485505	Fc receptor-like protein 5 isoform	1.4	-
LOC110516728	GTPase IMAP family member 4-like (1)	22.0	-
LOC110521965	GTPase IMAP family member 4-like (2)	7.6	-

While most immune related DEGs were downregulated in both phenotypes, the two most highly upregulated genes in resistant fish were homologs of GTPase IMAP family member 4-like at 22.0 and 7.6 log_2-FC, respectively. GIMAPs (GTPase of the immunity associated protein family) are a relatively recently described family of small GTPases that are conserved among vertebrates and are associated with T-lymphocyte development and activation (Filén and Lahesmaa, 2010). Two immune receptors were also upregulated in resistant fish: NLRC 5 and Fc receptor-like protein 5. In susceptible fish, only two immune genes were upregulated: B-cell receptor CD22-like and CD209 antigen-like protein E.

Intestine 7 dpe - resistant and susceptible fish - differential expression and GO enrichment

37,978 genes were identified in the intestine at 7 dpe. As for gills, DEGs were identified by comparing exposed fish to their unexposed controls. In contrast to the large number of DEGs in the gills at 1 dpe, only 16 DEGs were identified in resistant fish, 4 in susceptible fish, and no DEGs overlapped between them (Table 2.4). No GO enrichment was conducted due to the small number of DEGs.

Table 2.4. Genes that were differentially expressed in the intestine of resistant and susceptible steelhead 7 days post exposure to *Ceratonova shasta*. Genes with known immune functions are in bold. Non-significant differences in expression are marked as "-".

Entrez Gene ID	Protein Product	Log_2-FC	Log_2-FC
LOC110534740	**fucolectin 6**	**9.4**	-
LOC110492870	aginyl-tRNA--protein transferase 1	6.4	-
LOC110539108	battenin-like	6.2	-
LOC110534594	fibronectin-like	4.6	-
LOC110507973	myb/SANT-like DNA-binding domain-containing protein	4.4	-
LOC110487421	**protein NLRC5**	**3.3**	-
LOC110502432	**ras guanyl-releasing protein 3**	**3.3**	-
lncRNA	lncRNA 3 3	2.7	-
LOC110536765	uncharacterized protein LOC110536765	2.7	-
Ig kappa-b4 chain C region	**immunoglobulin kappa constant**	**1.9**	-
LOC110501851	isocitrate dehydrogenase e	-1.1	-
LOC110504050	WW domain-containing oxidoreductase	-1.1	-
LOC110507963	retinol-binding protein 2	-1.4	-
LOC100135970	toxin-1 precursor	-2.0	-
LOC110487883	1-acylglycerol-3-phosphate O-acyltransferase	-3.9	-
LOC110517324	desmin-like	-5.2	-
LOC110512982	protein CREG1-like	-	21.3
LOC110507394	angiopoietin 1	-	2.3
LOC110520527	uncharacterized protein LOC110520527	-	1.5
LOC110520289	trans-1,2-dihydrobenzene-1,2-diol dehydrogenase	-	-1.5

Among the DEGs in resistant fish that have known functions, four immune genes were upregulated, including two innate immune receptors: Fucolectin 6, an F-type lectin that binds fucose, and NLRC 5, which was also upregulated in the gills of resistant fish at 1 dpe. Two immune genes involved in B cell responses were also upregulated: Ras guanyl-released protein 3, involved in B cell activation (Aiba et al., 2004), and immunoglobulin kappa constant. Fibronectin-like, an extracellular matrix protein, and battenin-like were also significantly upregulated. Battenin, also called CLN3, is a highly conserved multi-pass membrane protein that localizes to the lysosome and other vesicular compartments, but the function of which remains unknown (Fossale et al., 2004). The most downregulated gene in resistant fish was desmin-like protein, a muscle specific intermediate filament.

In susceptible fish, the cell-growth inhibitor protein CREG1 was the most highly upregulated transcript, followed by the vascular growth factor angiopoietin-1-like.

Comparison of resistant and susceptible controls

To identify any genes involved in resistance to *C. shasta* that might be constitutively expressed in resistant fish, we conducted a differential gene expression analysis comparing the uninfected controls for both phenotypes. This yielded 1400 DEGs in the gills, and 307 in the intestine. 38 DEGs were present in both tissues and upregulated in resistant fish relative to susceptible fish (S2 Table). Among them were six genes associated with immune system functions: two homologs of NLRC 5 (not the same one upregulated in response to *C. shasta* infection), GTPase IMAP family member 7-like, complement C1q-like protein 2, TGF-beta receptor type-2-like, and perforin-1-like.

Intestine - susceptible fish - 14 and 21 dpe - differential gene expression and GO enrichment

The transcriptomic response of susceptible fish was followed through later timepoints to determine how these fish reacted as the parasite continued to proliferate. Sequencing of infected fish and their time-matched controls identified 36,957 and 36,346 gene transcripts at 14 and 21 dpe, respectively. Comparison to the intestine of uninfected susceptible fish revealed 5,656 DEGs at 14 dpe and 12,061 DEGs at 21 dpe, 3,708 of which were differentially expressed at both timepoints (Figure 2.6).

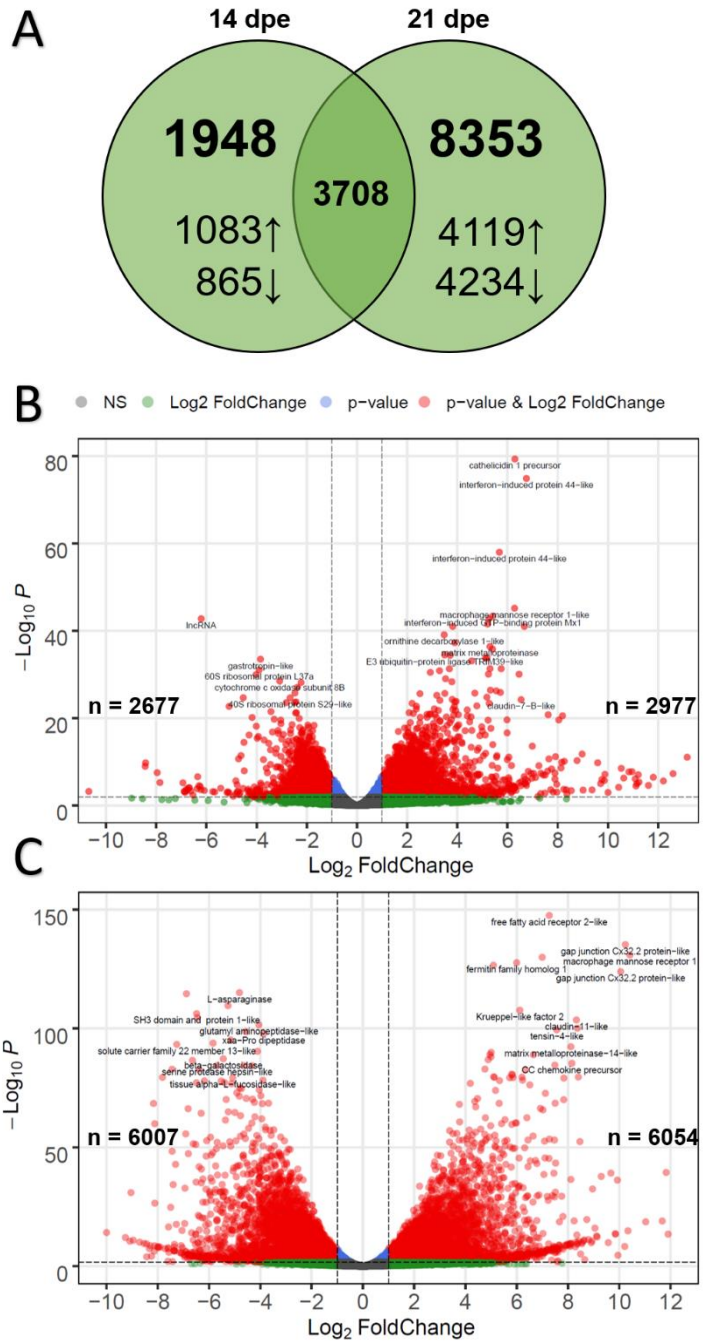

Figure 2.6. Differential expression results for susceptible fish at 14- and 21-days post exposure (dpe) to *Ceratonova shasta*. A) Venn diagram indicating the number of differentially expressed genes overlapping at 14- and 21 dpe. Arrows indicate up- vs. downregulation. B) Volcano plot of differential gene expression for susceptible fish at 14 dpe. Each dot represents the average value of one gene across three biological replicates. Red indicates the gene was significant at the FDR-adjusted p-value and Log2-Foldchange threshold, blue is significantly only by p-value, green only by Log2-Foldchange, and gray were not significant by either metric. B) Same as (A), but for susceptible fish at 21 dpe.

GO enrichment analysis of the 2,977 upregulated genes at 14 dpe indicated 631 over-represented GO terms, primarily immune related. ClueGO analysis clustered these into networks revolving around GO terms for interferon-gamma-mediated signaling pathway, regulation of defense response, positive regulation of response to external stimulus, immune response, and innate immune response (Figure 2.7A). The same analysis for the 2,677 downregulated genes at 14 dpe yielded 196 GO terms, which clustered into networks based on terms for oxidation-reduction process, mitochondrion organization, translation, and lipid catabolic process (Figure 2.7B).

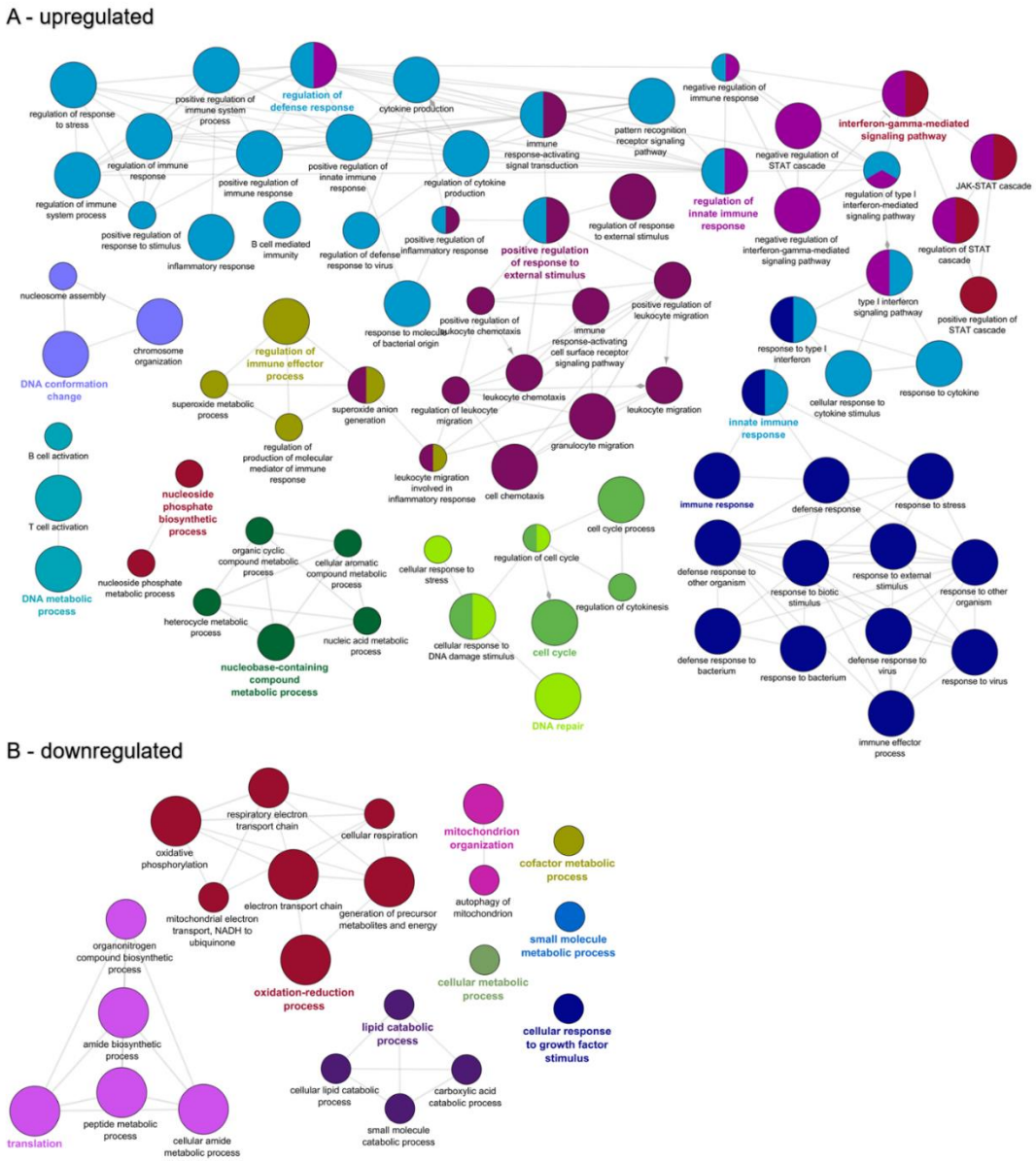

Figure 2.7. Functional enrichment of biological processes among the genes differentially expressed in the intestine of susceptible fish at 14 days post exposure to *Ceratonova shasta*. Enriched gene ontology (GO) terms were grouped into functionally related nodes using the Cytoscope plugin ClueGO. Nodes are colored and grouped according to a related function and labelled by the most significant term of the group. Node size corresponds to the FDR-adjusted p-value of each GO term and is specific to each graph. The analysis was conducted separately on upregulated (A) and downregulated (B) genes.

At 21 dpe, the 6,054 upregulated genes contained 452 over-represented GO terms which primarily clustered into networks revolving around immune system processes such as immune response-activating signal transduction, positive regulation of immune system process, immune response-activating cell surface receptor signaling pathway, and regulation of immune response (Fig 2.8A). In addition to these immune system pathways, cell adhesion pathways came to the forefront, including cell-matrix adhesion, cytoskeleton organization, integrin-mediated signaling pathway, and positive regulation of cell adhesion. The 6,007 downregulated genes were enriched for 152 GO terms that clustered into networks for lipid catabolic process, oxidation-reduction process, lipid metabolic process, and cofactor metabolic process (Fig 2.8B).

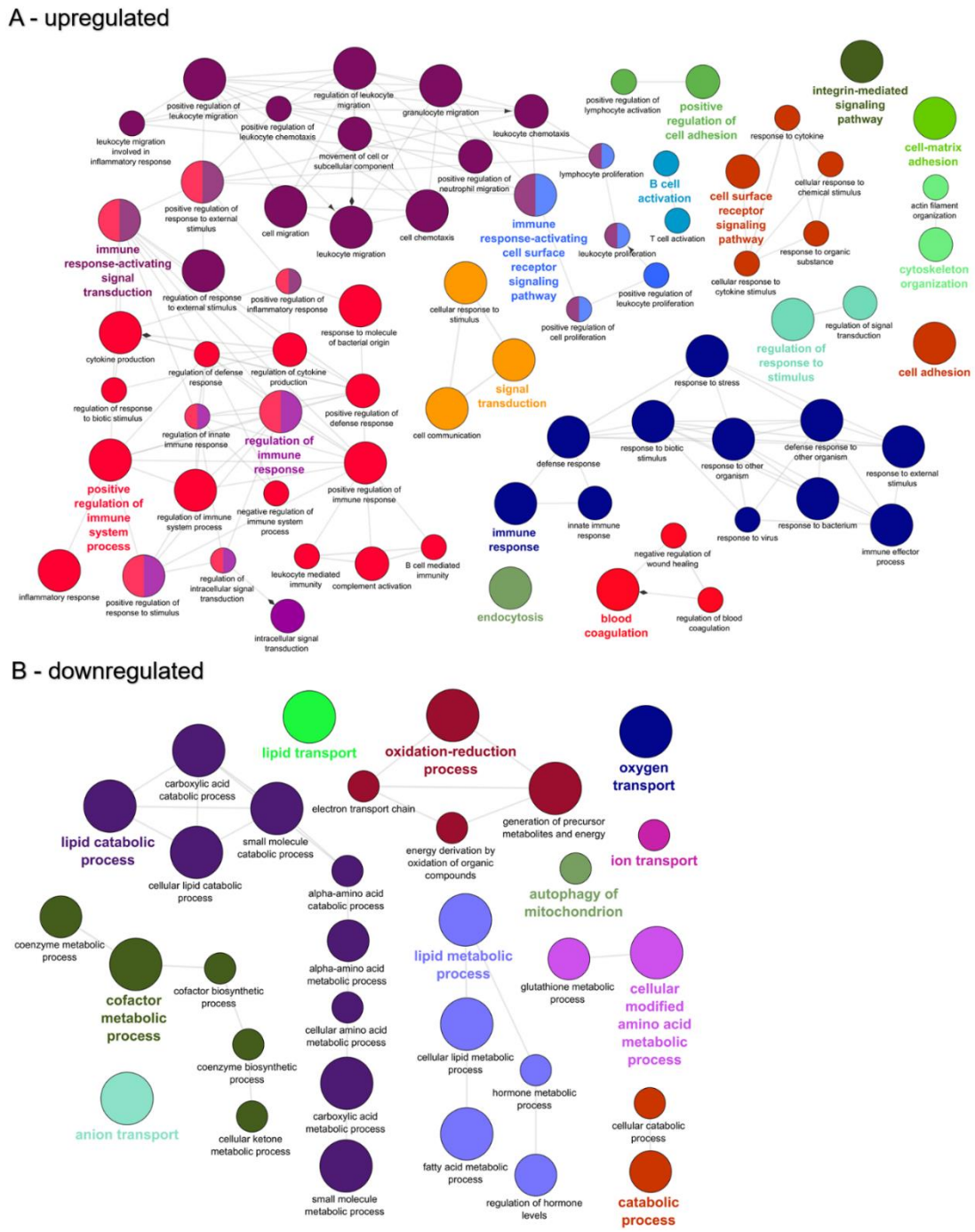

Figure 2.8. Functional enrichment of biological processes among the genes differentially expressed in the intestine of susceptible fish at 21 days post exposure to *Ceratonova shasta*. Enriched gene ontology (GO) terms were grouped into functionally related nodes using the Cytoscope plugin ClueGO. Nodes are colored and grouped according to a related function and labelled by the most significant term of the group. Node size corresponds to the FDR-adjusted p-value of each GO term and is specific to each graph. The analysis was conducted separately on upregulated (A) and downregulated (B) genes.

Key genes expressed in response to C. shasta infection in susceptible fish

Due to the large number of DEGs detected, only a subset of key genes identified in our analysis are presented in Table 2.5 and described below. The complete list of differential gene expression results and GO enrichment can be found in S2 Table.

Table 2.5. Select immune genes that were differentially expressed in the intestine of susceptible steelhead at 14- and 21-days post exposure (dpe) to *Ceratonova shasta*. Non-significant differences in expression are marked with "-".

Entrez Gene ID	Protein Product	Log_2-FC	Log_2-FC
Cytokines			
LOC100136024	interleukin-1 beta	3.3	-
LOC110536401	interleukin-1 beta-like	9.0	5.3
il-6	interleukin-6 precursor	-	5.9
LOC110496949	interleukin-6-like	7.5	10.3
il-8	putative CXCL8/interleukin-8	2.6	5.3
LOC110531606	CXCL8/interleukin-8-like	1.9	5.9
tnf	tumor necrosis factor	-	-
mif	macrophage migration inhibitory factor	-2.0	-2.7
LOC110488642	macrophage migration inhibitory factor-like	-2.2	-2.2
csf-3	granulocyte colony-stimulating factor	4.4	-
csf3r	granulocyte colony-stimulating factor receptor	1.6	3.0
Effector			
LOC110536463	granzyme A-like	5.2	2.7
LOC110520655	granzyme B-like	3.9	5.0
LOC110524258	granzyme-like protein 2	-	2.9
LOC110531658	perforin-1-like	1.7	1.4
LOC110536422	perforin-1-like	-1.7	-1.2
LOC110538116	perforin-1-like	3.9	7.4
LOC110500520	perforin-1-like	-	5.0
LOC100136187	cathelicidin antimicrobial peptide	5.8	8.3
LOC100136204	cathelicidin 1 precursor	6.3	7.5
LOC110523157	lysozyme C II	-	2.5
LOC110485102	lysozyme g-like	-	2.0
Macrophages			
LOC110498289	arginase-2, mitochondrial-like	-	4.1
LOC110506002	arginase-2, mitochondrial-like	4.1	4.0
nos2	nitric oxide synthase, inducible	-	-
LOC110507147	nitric oxide synthase, inducible (Fragment)-	4.7	-
LOC110536912	macrophage mannose receptor 1-like	-1.9	-6.7
LOC110500089	macrophage mannose receptor 1-like	-	-3.9
LOC110508265	macrophage mannose receptor 1-like	6.3	10.4

LOC110508267	macrophage mannose receptor 1-like	5.5	7.8
LOC110516203	macrophage mannose receptor 1-like	5.0	8.2
T_{H1} response			
ifng	interferon gamma	-	6.6
ifngamma2	interferon gamma 2	5.4	4.7
ifngr1	interferon gamma receptor 1	4.2	3.5
ifngr1	interferon gamma receptor alpha chain	-	1.6
irf-8	interferon regulatory factor 8-like	3.7	2.5
il12b	interleukin-12 beta chain precursor	2.1	2.2
LOC110537792	interleukin-12 subunit beta-like	-	-4.0
LOC110524480	interleukin-12 receptor subunit beta-2-like	1.1	-1.3
LOC110524481	interleukin-12 receptor subunit beta-2-like	1.5	2.9
LOC110511354	interleukin-18 receptor accessory protein-like	-	5.0
tbx21	T-bet	2.4	4.3
stat1-1	signal transducer/activator of transcription 1	2.1	1.3
LOC110520020	signal transducer and activator of transcription 1-alpha/beta-like	3.8	3.0
LOC110501544	signal transducer and activator of transcription 1-alpha/beta-like	2.5	3.2
T_{H2} response			
il4/13a	interleukin-4/13A precursor	5.4	5.0
LOC110489171	interleukin-4/13b1 precursor	6.1	5.0
LOC110504551	interleukin-4/13b2 precursor	7.9	7.7
il17c1	interleukin-17C1 precursor	-	-6.8
LOC110492428	interleukin-17 receptor C-like	-	-2.7
socs3	suppressor of cytokine signaling 3	4.1	4.1
LOC110512513	suppressor of cytokine signaling 3-like	3.9	3.9
LOC110500122	transcription factor GATA-3-like	-	2.2
T_{H17} response			
il-17a	interleukin-17A precursor	-8.4	-6.7
LOC110504334	interleukin-17A-like	-	-5.4
LOC110529296	interleukin-17A-like	1.4	-
il-17d	interleukin-17 isoform D precursor	-2.1	-
LOC110497745	interleukin-17F-like	-	-2.0
LOC110505720	interleukin-17F-like	-	-8.3
il17rd	interleukin-17 receptor D	-	-1.2
LOC110492331	interleukin-17 receptor D-like	1.4	2.0
il17r	interleukin-17 receptor precursor	-	-2.4
il-22	interleukin-22 precursor	-	-3.1
LOC110524663	interferon regulatory factor 4-like	1.3	1.4
LOC110538194	signal transducer and activator of transcription	-	-1.2
LOC110520784	nuclear receptor ROR-gamma-like	-	-2.8
LOC110535950	nuclear receptor ROR-gamma-like	-	-2.4
T_{reg} response			
il10	interleukin-10 precursor	6.3	8.1

il10b	interleukin-10b protein precursor	4.8	6.0
LOC100136774	transforming growth factor beta-1	1.4	1.4
LOC110534057	transforming growth factor beta-1-like	1.7	3.9
tgfb1i1	transforming growth factor beta-1-induced	-	2.4
foxp3-1	forkhead box P3-1 protein	-	-1.9
foxp3-2	forkhead box P3-2 protein	-	-2.3
B cell response			
LOC110522002	Blimp-1/PR domain zinc finger protein 1-like	4.9	7.5
LOC110496128	Blimp-1/PR domain zinc finger protein 1-like	3.3	4.2
LOC110485501	B-cell receptor CD22-like	2.5	5.2
LOC110538709	immunoglobulin heavy variable 1-69-2-like	5.7	8.0
LOC110490545	Ig kappa chain V region K29-213-like	2.3	2.4
LOC110535024	immunoglobulin kappa light chain-like	2.1	2.1

Cytokines

The pro-inflammatory cytokine interleukin-1 beta (IL-1β) was highly upregulated at 14- and 21 dpe. IL-1β is a chemoattractant for leukocytes in fish and modulates the expression of other chemokines including CXCL8/interleukin-8 (Secombes et al., 2011), which was also upregulated at both timepoints. Curiously, the pro-inflammatory cytokine TNA-α was not differentially expressed at either timepoint despite the upregulation of other pro-inflammatory cytokines, including IL-1β which stimulates the production of TNA-α. Macrophage migration inhibitory factor (MIF) is a pro-inflammatory cytokine that acts as a mediator of both innate and acquired immunity. It is implicated in resistance to bacterial pathogens and is released from macrophages after stimulation with LPS. Mice that lack MIF are more susceptible to leishmaniasis and cysticercosis and *in vivo* administration of recombinant MIF reduced the severity of *Leishmania major* pathogenesis in mice (Calandra and Roger, 2003). We observed downregulation of two MIF homologs at both 14 and 21 dpe.

Effector enzymes

We detected low to high upregulation of several granzyme and perforin transcripts at 14- and 21 dpe. Cytotoxic T lymphocytes (CTLs) release these proteins in secretory granules to induce apoptosis of infected or damaged cells. The antimicrobial peptide cathelicidin was highly upregulated at both timepoints, while lysozyme was upregulated only at 21 dpe.

Macrophage activation and polarization

Macrophages at the site of inflammation polarize into M1 or M2 phenotypes. M1 polarization is associated with the T_{H1} response and the presence IFN-γ and induces macrophages

to express the enzyme nitric oxide synthase (NOS) leading to the production of reactive nitrogen species for pathogen clearance. M2 polarization is driven by the T_{H2} response and the presence IL-4/13. M2 macrophages are associated with wound healing and the expression of the arginase enzyme. We observed upregulation of NOS at 14 dpe but not at 21 dpe. The opposite was true for arginase, which was only upregulated at 21 dpe.

Macrophage mannose receptor 1 (MCR1) is a transmembrane glycoprotein belonging to the C-type lectin family. In addition to scavenging certain hormones and glycoproteins, it also recognizes a variety of pathogens including influenza virus, *Yersinia pestis*, and *Leishmania* species (Azad et al., 2014). Ten homologs of MCR1 were differentially expressed at 14- or 21 dpe and were among the most highly induced immune genes at 21 dpe.

GTPase IMAP family members

A total of 15 GIMAP proteins were upregulated at 14 dpe, including two homologs of GTPase IMAP family member 4-like which were the two most highly upregulated immune genes at this timepoint (10.4 and 9.0 log_2-FC). The same two homologs were also the most highly upregulated genes (22.0 and 7.6 log_2-FC) in the gills of resistant fish at 1 dpe. However, they were not differentially expressed in susceptible fish at 21 dpe. At 21 dpe, only five GIMAPs proteins were upregulated, with GTPase IMAP family member 7-like having the highest increase in expression (4.1 log_2-FC).

Activated T-cells

$CD4^+$ T helper cells (T_H cells) are an important wing of the adaptive immune response that differentiate into one of several effector subsets (T_{H1}, T_{H2}, T_{H17}, and T_{reg}) based on the cytokine signals they receive. These effector cells, in turn, secrete their own district profile of cytokines that help orchestrate the immune response. Among the genes differentially expressed in response to *C. shasta* infection, signature genes for each subset were identified to provide insight into the T cell response (Table 2.6).

Interferon gamma (IFN-γ), the signature T_{H1} cytokine, was highly upregulated at both 14- and 21 dpe along with its cognate receptor and T-bet, the master transcriptional regulator of T_{H1} differentiation. Only one gene related to interleukin-12, the primary driver of T_{H1} differentiation, was upregulated at 14 dpe (interleukin-12 subunit beta-like, 2.3 Log_2-FC). The gene was similarly upregulated at 21 dpe, along with interleukin-12 alpha and beta chains.

Interleukin-4/13 is the primary cytokine produced by T_{H2} cells and drives alternative macrophage activation and type 2 inflammation. Moderate upregulation of interleukin-4/13A precursor was seen at 14- and 21 dpe. The master transcriptional regulator of T_{H2} differentiation, GATA-3, was only upregulated at 21 dpe.

Downregulation of several genes involved in the T_{H17} response was observed at 14- and 21 dpe. Most significant of these was interleukin-17A precursor, and interleukin-17F-like. Two copies of nuclear receptor ROR-gamma, the putative master transcriptional regulator of T_{H17}, were downregulated at 21 dpe.

Little evidence of a strong regulatory T cell response was seen at either 14- or 21 dpe. FOXP3, the master transcriptional regulator for T_{reg} cells, was downregulated at 21 dpe. Transforming growth factor beta transcripts were mildly upregulated at both timepoints. Interleukin-10, which is classically associated with T_{reg}, was highly upregulated at 14- and 21 dpe, however, it can be produced by numerous different myeloid and lymphoid cells during an infection (Couper et al., 2008). This lack of an observable T_{reg} response may be due to the significant upregulation of interleukin-6 seen at both timepoints, as interleukin-6 is known to inhibit T_{reg} conversion in humans and mice (Korn et al., 2008; Goodman et al., 2009).

B cell response

Numerous genes involved in the B cell response and production of immunoglobulins were upregulated at 14 dpe, and both the number of genes and the magnitude of the upregulation increased at 21 dpe. Among these were the transcription factor Blimp-1, which is required for the maturation of B cells into Ig-secreting cells, B cell receptor CD22, and several heavy and light chain transcripts.

Innate immune receptors

Toll-like receptors (TLRs) are innate immune receptors that recognize conserved pathogen-associated molecular patterns. We observed upregulation of six different TLRs at 14- or 21 dpe, including eleven homologs of TLR13. In mice, TLR13 recognizes a conserved bacterial 23S ribosomal RNA sequence, a function that appears to be conserved in teleost fish (Wang et al., 2016). Two copies of TLR8, which recognizes viral single-stranded RNA, were upregulated at both timepoints, and one copy of TLR1, which recognizes bacterial lipoprotein, and TLR22. TLR22 is a fish-specific TLR and has been shown to be induced after viral, bacterial, or ectoparasite challenge (Panda et al., 2014). TLR3 and TLR7, which recognize viral

RNA, were upregulated at 14 dpe. Although they were different homologs than those upregulated in resistant fish, 18 putative NOD-like receptors were upregulated at 14 dpe and 11 at 21 dpe. We also observed substantial upregulation of C-type lectins, with 16 upregulated at 14 dpe and 20 upregulated at 21 dpe.

Cell adhesion

Genes involved in cell-to-cell contact and the formation of the intestinal barrier were among the most transcriptionally active at both timepoints, with the majority of transcripts being upregulated. At 14 dpe, this included 10 claudins, 19 integrins, 1 fibronectin, 5 fermitin family homologs, 8 gap junction proteins, and 17 cadherins. This continued at 21 dpe with 23 claudins, 42 integrins, 11 fibronectins, 7 fermitin family homologs, 15 gap junction proteins and 36 cadherins. Additionally, in terms of statistical significance, the actin binding protein beta-parvin was the most significant DEG at 21 dpe (padj = 9.97e-232, $\log_2$-FC = 7.6).

Validation of DEGs using RT-qPCR

Four immune genes (*IFN-γ, TNF-α, IL-10, IL-1β)* found to be differentially expressed by RNA-seq were assayed using quantitative reverse transcription PCR (RT-qPCR) to validate the results and confirm the observed downregulation of immune genes. Fold changes from RT-qPCR are compared with those from RNA-seq in Figure 2.9 and support the results we obtained.

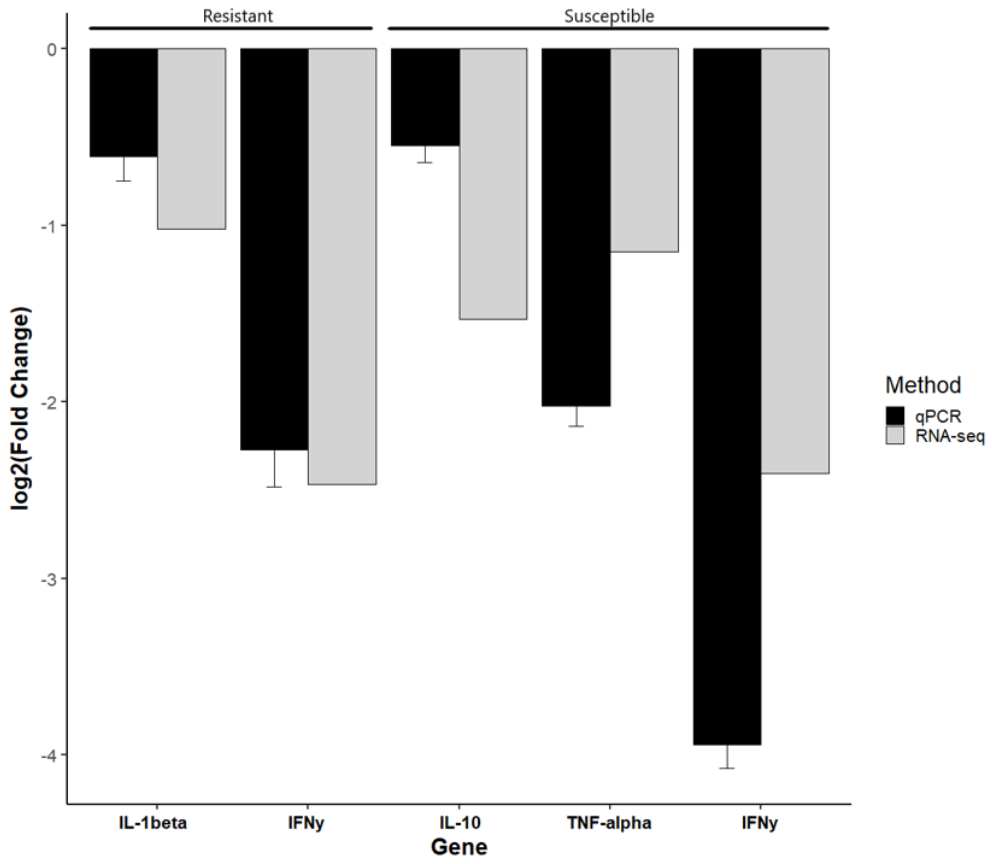

Figure 2.9. qPCR validation of RNA-seq results. Quantitative reverse transcription PCR (RT-qPCR) validation of four immune genes (IFN-γ, TNF-α, IL-10, IL-1β) found to be significantly differentially expression by RNA-seq at day 7 in the gills. The X-axis shows the gene and phenotype assayed and the Y-axis shows the relative log2(Fold Change) between fish exposed to *Ceratonova shasta* and their respective control. Error bars indicate the standard deviation of Cq values between biological replicates. Continued on next page.

DISCUSSION

We used RNA-seq to study the early transcriptomic response of resistant and susceptible steelhead infected with the myxozoan parasite *C. shasta*. Comparative transcriptomics revealed that both phenotypes had a suppression of the interferon gamma signaling pathway in the gills at 1 dpe. The response of the two phenotypes quickly diverges after that. In the intestine at 7 dpe, resistant fish had effectively contained the parasite and several immune genes were upregulated in this tissue. Susceptible fish, on the other hand, had no observable response to parasite proliferation in the intestine at this time. Parasite replication in susceptible fish continued

exponentially at 14- and 21 dpe, which coincided with an intense, yet ineffective immune response and the breakdown of the intestinal structure.

Immunosuppression at the portal of entry (gills)

Given the markedly different resistance of these two fish stocks to *C. shasta* induced pathological changes, the overall transcriptomic response in the gills was surprisingly similar, with a downregulation of immune genes in both phenotypes. We observed a suppression of the innate immune response, particularly the IFN-γ signaling pathway which is the primary immune pathway activated later in the infection. This may reflect a parasite-induced immunosuppression that aids in initial invasion of the host. Immunosuppression is a well-known method of immune evasion for human parasites (Zambrano-Villa et al., 2002), and an immunosuppressed state has been observed in other fish-parasite systems, including infections by other myxozoans. A microarray analysis of gilthead sea bream exposed to the myxozoan *Enteromyxum leei* revealed that successfully parasitized fish were characterized by a global downregulation of genes involved in the immune and acute phase response (Davey et al., 2011). Studies of rainbow trout infected with the related malacosporean *Tetracapsuloides bryosalmonae*, the causative agent of proliferative kidney disease, revealed suppression of phagocytic activity and oxidative burst (Chilmonczyk et al., 2002), and a dysregulated T-helper and B cell response (Abos et al., 2018; Gorgoglione et al., 2013). The transcriptomic response of Atlantic salmon affected by amoebic gill disease, caused by a protozoan parasite, is also associated with downregulation of immune genes, including those related to MHC I and IFN-γ (Wynne et al., 2008; Young et al., 2008).

Potential recognition of the parasite by resistant fish

Although the majority of immune genes were downregulated in the gills of resistant fish, two copies of GTPase IMAP family member 4-like were the most highly upregulated genes at this timepoint. Additionally, the immune receptors NLRC5 and Fc receptor-like protein 5 were also upregulated. The upregulation of innate immune receptors, including NLRC 5 which was also upregulated in the intestine of resistant fish, suggests that specific recognition of *C. shasta* may be occurring in these fish. While this may not offer protection at the portal of entry, it may enable a more rapid immune response to the parasite at the intestine, or during its migration there. This would explain why resistant fish had a much lower infection prevalence and intensity in the intestine (Fig 2.3).

GIMAPs may mediate resistance to C. shasta

As noted above, the two most highly upregulated genes in the gills of resistant fish were two homologs of GTPase IMAP family member 4-like, a protein involved in T-lymphocyte development. Intriguingly, the same two homologs were the most highly upregulated immune genes in the intestine of susceptible fish at 14 dpe. If these genes are involved in mediating resistance to *C. shasta*, then their delayed expression in susceptible fish could explain the delayed immune response observed in these fish. How these genes might mediate resistance is unclear, as their precise function remains unknown. One possible mechanism may be through mediating the effects of IFN-γ, which orchestrates a plethora of cellular pathways and regulates the expression of hundreds of genes. In mice, IFN-γ driven pathogen resistance is dependent on certain families of GTPases (Boehm et al., 1998; Pilla-Moffett et al., 2016). Resistance to *Toxoplasma gondii* requires IFN-γ and it was recently shown that GIMAP proteins mediate resistance to *T. gondii* infection in the resistant Lewis rat strain, with overexpression of GIMAPs in rat macrophages showing that GIMAP 4 had the highest inhibitory effect (Kim et al., 2018).

Differences in parasite recognition in the intestine of resistant and susceptible fish

The lack of a transcriptomic response, including any upregulation of immune genes, in the intestine of susceptible fish at 7 dpe was surprising given the high parasite load present in this tissue at that time (Fig 2.3), and that initial invasion would have occurred 2-3 days prior (Bjork and Bartholomew, 2010). This would indicate that susceptible fish are unable to recognize the parasite invading the intestine or the subsequent proliferation. In contrast, resistant fish were able to either prevent parasite establishment in the intestine or minimize parasite proliferation once there. Consistent with this, we observed upregulation of several immune genes in resistant fish. Immunoglobulin kappa constant, which encodes the constant region of immunoglobulin light chains, was mildly upregulated. Fucolectin 6, an F-type lectin that binds fucose was highly upregulated at this timepoint. Lectins are carbohydrate-binding proteins that play a key role in the innate immune response by recognizing exposed glycans on the surface on pathogens (Vasta et al., 2017). We also observed upregulation of the same homolog of NLRC 5 that was upregulated in the gills of resistant fish at 1 dpe. NOD-, LRR- and CARD-containing (NLRC) proteins are a group of pattern recognition receptors that play a role in both innate and adaptive immune responses by inducing transcription of pro-inflammatory and MHC class I genes, and triggering formation of the “inflammasome”, a multi-protein complex that results in

programmed cell death (Benkő et al., 2017; Zhao and Shao, 2012). NLRCs are known to play a role in the mucosal immune system of the mammalian gut and are highly expressed by macrophages and epithelial cells in the intestine (Chassaing et al., 2014). Numerous studies of teleost fish have demonstrated the presence of NLRCs that are induced upon immune stimulation or exposure to a pathogen (Laing et al., 2008; Sha et al., 2009; Chang et al., 2011; Hou et al., 2012; Park et al., 2012; Rajendran et al., 2012; Swain et al., 2012; Li et al., 2012; Xie et al., 2013). With the generation of several high quality teleost genomes, it is evident that a shared expansion of NLRC genes has occurred in teleosts, suggesting a more prominent role in the immune system (Tørresen et al., 2018). Considering that myxozoans predate the evolution of fish and have been co-evolving with their acquired vertebrate hosts for hundreds of millions of years (Holzer et al., 2018), it seems plausible that fish would have evolved innate immune receptors capable of recognizing conserved motifs on these ubiquitous pathogens.

Susceptible fish exhibit a vigorous yet ineffective T_{H1} response

Evidence of a strong T_{H1} response was observed in susceptible fish at both 14 and 21 dpe, with upregulation of IFN-γ, its cognate receptor, and T-bet, the master transcriptional regulator of T_{H1} differentiation. GO enrichment analysis also revealed that genes involved in the interferon-gamma signaling pathway were over-represented among the upregulated genes. Upregulation of IFN-γ has been observed in previous studies of Chinook and rainbow trout exposed to *C. shasta* (Bjork et al., 2014; Hurst et al., 2019; Taggart-Murphy, 2018) and appears to play a pivotal and conserved role in the fish response to myxozoan infections. Studies of resistant and susceptible rainbow trout exposed to the myxozoan *Myxobolus cerebralis*, the causative agent of whirling disease, have shown a strong induction of IFN-γ and interferon regulatory factor 1 in both strains, with IFN-γ being upregulated earlier in the infection in resistant fish (Baerwald, 2013; Baerwald et al., 2008). Olive flounder (*Paralichthys olivaceus*) infected with the myxozoan *Kudoa septempunctata* had elevated levels of IFN-γ in their trunk muscles (Jang et al., 2017). IFN-γ was also found to be upregulated in turbot during the early stages of enteromyxosis caused by *E. scophthalmi* (Ronza et al., 2016). Most interestingly, when gilthead sea bream (*Sparus aurata L.*) were exposed to *E. leei*, only the non-parasitized fish had elevated levels of IFN-γ, suggesting it helps mediate resistant to the pathogen (Davey et al., 2011).

If the IFN-γ pathway is a primary way of defending against myxozoan infections, it raises the question as to why it's activation in susceptible fish offered no apparent protection against *C. shasta* pathogenesis. Bjork et al. (Bjork et al., 2014) suggest that upregulation of the potent anti-inflammatory cytokine IL-10 in susceptible fish may attenuate their inflammatory response and subsequent ability to control parasite proliferation. In concordance with that, we observed marked upregulation of several IL-10 homologs at both timepoints. The ability of IL-10 to attenuate IFN-γ driven parasite clearance by inhibiting the activity of macrophages, T_{H1} cells, and natural killer cells is well-documented (Couper et al., 2008; Gazzinelli et al., 1992; Netea et al., 2004). These immunosuppressive effects are exploited by certain pathogens, including koi herpesvirus, which encodes and expresses a functional IL-10 homolog (Sunarto et al., 2012). Dysregulation of IL-10 production, in terms of timing or over-expression, may explain why susceptible fish fail to inhibit parasite proliferation despite upregulation of IFN-γ.

The breakdown of the intestinal barrier in susceptible fish

The mucosal surface of the intestine must function as a site of nutrient absorption while acting as a barrier against the systemic spread of microorganisms, both commensal and pathogenic. The main physical component of the intestinal barrier is formed by a continuous monolayer of cells tightly attached to each other by tight junctions, adherens junctions, and desmosomes. Breakdown of this barrier can result in the systemic spread of harmful bacteria and molecules. *C. shasta* reaches the intestine via blood vessels and then migrates through the tissue layers to release spores into the intestinal lumen. As recently shown by Alama-Bermejo et al. (Alama-Bermejo et al., 2019), *C. shasta* genotype II is highly mobile and has strong adhesive affinities for the glycoprotein components of the extracellular matrix (ECM), resulting in massive interaction and disruption of the host intestinal ECM. We found that genes related to the ECM and cell adhesion showed an intense amount of transcriptional activity in susceptible fish at both 14- and 21 dpe. This aligns with the breakdown of the intestinal structure we observed in histological sections of these fish (Fig. 2.2A-C). Disrupted cell adhesion and cell-to-cell contact also interferes with intercellular communication through gap junctions, which is critical for maintaining tissue structure and homeostasis. Additionally, it can also lead to anoikis, a form of programmed cell death that occurs upon detachment from the ECM. The inability of susceptible fish to overcome *C. shasta* induced breakdown of the ECM would explain why we don't observe an organized tissue response to the infection (granulomas, fibrosis), as observed in resistant fish.

It is likely that this disruption of the host intestinal barrier and ECM in susceptible fish also lead to the dissemination of bacteria into the intestinal tissue, as evidenced by the upregulation of numerous toll-like receptors that recognize bacterial motifs, as well as cathelicidins, lysozyme, and complement proteins. Pathway level analysis showed the overall immune response transitioned from being primarily IFN-γ driven at 14 dpe (Fig 2.7A), to a more mixed immune response at 21 dpe (Fig. 2.8A). This likely influx of bacteria coincided with the downregulation of T_{H17} markers IL-17A, IL-17F, and ROR-gamma. T_{H17} cells play a critical role in the response to bacterial pathogens at the gut mucosal surface, and the expression of IL-17A and IL-17F generally increases after exposure to an intestinal pathogen (Blaschitz and Raffatellu, 2010; Kolls and Khader, 2010; Zhang et al., 2014). It should also be noted that IL-17F was also downregulated in the gills of susceptible fish at 1 dpe. Whether this represents a maladaptive host response, or a pathogenic strategy remains to be determined. However, it has been shown that certain pathogens actively interfere with the host IL-17 pathway. The mucosal pathogen *Candida albicans* inhibits IL-17 production in human hosts, which is the primary pathway for elimination of the fungus (Cheng et al., 2010), and the intracellular bacteria *Coxiella burnetii* blocks IL-17 signaling in human macrophages (Clemente et al., 2018).

In addition to the likely dissemination of bacteria caused by the breakdown of the intestinal barrier, the hosts ability to acquire nutrients and produce energy became severely compromised. The downregulated genes at 14- and 21 dpe primarily clustered around metabolic and energy producing pathways (Fig. 2.7B, 2.8B). This occurs while the host is trying to mount a massive immune response, an energetically costly endeavor. This highlights the uphill battle that susceptible fish face: their delayed response to *C. shasta* means they must overcome an evolutionarily well-adapted pathogen that has replicated extensively, while doing so under metabolic stress and with a compromised intestinal structure.

CONCLUSIONS

The primary goal of this study was to determine if susceptible fish recognized *C. shasta* during the initial stages of infection. It is clear from the results at 7 dpe that they fail to recognize the parasite invading the intestine. We specifically used RNA-seq with a high number of replicates to give us the widest possible chance of seeing any genes that respond to the infection, but none were detected. Whether susceptible fish recognize *C. shasta* in the gills remains unclear. We detected a transcriptomic response to the infection; however, this may be actively

induced by the parasite and not by host recognition. The observation that both the sympatric (resistant) and allopatric (susceptible) hosts exhibited a similar gill response, and that susceptible fish had no response in the intestine at 7 dpe, supports the idea that the transcriptomic response is driven by the parasite and not by specific host recognition.

The second goal of this study was to identify putative *C. shasta* resistance genes, particularly innate immune receptors that could initiate the immune response. We observed upregulation of a NOD-like receptor whose elevated expression coincided with initial invasion of the gills and intestine. We also observed strong induction of two homologs of GTPase IMAP family member 4 in the gills of resistant fish and later on in the intestine of susceptible fish. Our laboratory is currently in the process of creating a QTL cross of *C. shasta*-resistant and susceptible *O. mykiss* to identify the genomic loci responsible for resistance. Locating these putative resistance genes within the identified loci would offer robust support for their involvement in *C. shasta* resistance and provide a potential marker for rapid identification of resistant fish stocks.

While not an initial goal of this study, we characterized the intestinal response of susceptible fish during the middle and late stages of *C. shasta* infection. As expected from previous studies of *C. shasta* and other myxozoan infections, the immune response was characteristic of an IFN-γ driven T_{H1} response. This response failed to offer any protection though, possibly due to excessive or mistimed expression of IL-10, or the suppression of the T_{H17} response. Comparing the intestinal response of susceptible fish to that of resistant fish with a similar *C. shasta* burden would help answer this, and identify what a successful immune response to the parasite looks like once it has invaded the intestine and begun to replicate.

C. shasta is an important pathogen of salmonid fish in the Pacific Northwest and has had an outsized impact on the Klamath River Basin fisheries. As for most myxozoans, what the parasite does within the host and how the host responds has largely remained a black box. The work presented here helps shed light on this process. More broadly, it improves our understanding of myxozoan-host interactions and in conjunction with other studies, may allow general patterns to emerge regarding the fish host's response. One such pattern may be the conserved adaption of IFN-γ to combat myxozoan infections. This immediately raises the question of how a pathway that is classically associated with the immune response to

intracellular pathogens mediates resistance to extracellular myxozoan parasites. Finally, we have identified putative resistance genes that can provide a starting point for future functional studies.

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CHAPTER 3: TRANSCRIPTOMIC AND MORPHOLOGIC PROFILING REVEALS TISSUE REPAIR MECHANISMS UNDERLYING RESISTANCE TO THE PARASITE *CERATONOVA SHASTA*

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ABSTRACT

Myxozoan parasites infect fish worldwide causing significant disease or death in many economically important fish species, including rainbow trout and steelhead (*Oncorhynchus mykiss*). The myxozoan *Ceratonova shasta* is a parasite of salmon and trout that causes ceratomyxosis, a disease characterized by severe inflammation in the intestine resulting in hemorrhaging and necrosis. Populations of *O. mykiss* that are genetically fixed for resistance or susceptibility to ceratomyxosis exist naturally, offering a tractable system for studying the immune response to myxozoans. The aim of this study was to understand how steelhead that are resistant to the disease respond to *C. shasta* once it has become established in the intestine and identify potential mechanisms of resistance.

Sequencing of intestinal mRNA from resistant steelhead with severe *C. shasta* infections identified 417 genes differentially expressed during the initial stage of the infection. A strong induction of interferon-gamma and interferon-stimulated genes was evident, along with genes involved in cell adhesion and migration. 11,984 genes were differentially expressed during the late stage of the infection, most notably interferon-gamma, interleukin-6, and immunoglobulin transcripts. A distinct hardening of the intestinal tissue and a strong inflammatory reaction in the intestinal submucosa including severe hyperplasia and inflammatory cell infiltrates were observed in response to the infection. The massive upregulation of caspase-14 early in the infection, a protein involved in keratinocyte differentiation might reflect the rapid onset of epithelial repair mechanisms, and the collagenous stratum compactum seemed to limit the spread of *C. shasta* within the intestinal layers. These observations could explain the ability of resistant fish to eventually recover from the infection.

Our results suggest that resistance to *ceratomyxosis* involves both the rapid induction of key immune factors as well as a tissue level response that limits the spread of the parasite and the subsequent tissue damage. These results improve our understanding of the myxozoan-host dialogue and provide a framework for future studies investigating the infection dynamics of *C. shasta* and other myxozoans.

INTRODUCTION

Myxozoans are parasitic cnidarians characterized by a complex two-host, two-spore lifecycle that alternates between an invertebrate host (annelids or bryozoans) and a vertebrate host (generally fish) (Kent et al., 2001). Myxozoans are widely distributed with over 2,400

known species, and have adapted to both freshwater and marine hosts (Zhang, 2011). Infections in the fish host are mostly asymptomatic, however, certain myxozoans are known to cause severe pathological changes or death of the fish host. Mortality and morbidity as a result of myxozoan infections is becoming more common as aquaculture continues to expand worldwide, and more fish species become intensively managed by humans (Sitjà-Bobadilla, 2008). For example, throughout Asia and the Mediterranean, cultivation of turbot (*Scophthalmus maximus*) and several perciform fish is severely limited by enteromyxosis, caused by the myxozoan parasites *Enteromyxum scophthalmi* and *E. leei*, respectively (Sekiya et al., 2016; Sitjà-Bobadilla and Palenzuela, 2012). Similarly, pharyngeal myxobolosis caused by *Myxobolus hunghuensis* is a limiting factor in the development of gibel carp (*Carassius auratus gibelio)* aquaculture in China (Zhao et al., 2019). Globally, over 35 species of marine fish are affected by *Kudoa thyrsites*, which encysts in muscle and causes postmortem myoliquefaction of the surrounding tissue, resulting in large economic losses due to poor fillet quality (Braden et al., 2017).

Rainbow trout, and the anadromous form steelhead (*Oncorhynchus mykiss*), are among the most widely cultivated species of fish and are negatively affected by several myxozoan pathogens, including *K. thyrsites*, *M. cerebralis (*the causative agent of whirling disease) (Sarker et al., 2015), and *Tetracapsuloides bryosalmonae,* which is a related malacosporean and causes proliferative kidney disease (Bailey et al., 2019). Within the Pacific Northwest of the United States, *O. mykiss* and related salmonids are severely impacted by the intestinal myxozoan *Ceratonova shasta* (syn. *Ceratomyxa shasta*) which causes ceratomyxosis, a disease characterized by hemorrhaging and necrosis of the intestine and potentially death of the fish host. *C. shasta* has been linked to population level declines of Pacific Northwest fish stocks (Fujiwara et al., 2011; Hallett et al., 2012), and exerts such a selective pressure on the fish host that endemic populations became genetically fixed for resistance to the disease (Bartholomew et al., 2001; Buchanan et al., 1983; Hemmingsen et al., 1986; Ibarra et al., 1992, 1994; Nichols et al., 2003). However, the parasite is not established in all river systems and allopatric salmonids are highly susceptible to the parasite, with a single spore capable of causing mortality (Bjork and Bartholomew, 2009; Ratliff, 1983). This leads to an almost binary resistance phenotype, where mortality either occurs after exposure to one spore, or thousands (Hallett et al., 2012; Ray et al., 2010).

The combination of a highly virulent myxozoan, along with naturally occurring resistant and susceptible strains of the fish host, offers an attractive system for studying the immune response to myxozoan parasites. Our research group previously conducted a comparative transcriptomic analysis of resistant and susceptible phenotypes of steelhead, exposed to a low dose of *C. shasta*, sufficient to cause mortality in the susceptible phenotype (chapter 2). RNA-seq analysis revealed downregulation of genes involved in the interferon-gamma signaling pathway in the gills, the site of initial parasite invasion, of both phenotypes at 1-day post exposure (dpe) to C. *shasta*. By 7-dpe, resistant fish had effectively contained the infection, having either low or undetectable levels of *C. shasta* in their intestine, with several immune genes upregulated. In contrast to the resistant fish, susceptible fish had a significant parasite burden in their intestine, but no genes with known immune functions were upregulated, highlighting a lack of early recognition in these fish. The parasite continued to replicate exponentially in susceptible fish, and sequencing of intestinal mRNA from these fish at 14- and 21-dpe revealed an interferon-gamma driven T_H1 response and a suppression of the T_H17 response. This intense T_H1 response failed to ameliorate the progress of the disease and the intestines of these fish progressively deteriorated. A comparable dataset from resistant fish with a similar parasite burden in their intestine could help us discern why the susceptible fishes' immune response failed to offer any protection.

Thus, in order to understand how fish with a resistant phenotype respond to the parasite once it becomes established in the intestine, we challenged resistant steelhead with a dose of *C. shasta* sufficient to result in pathological changes. Accomplishing this task in the laboratory presented a significant hurdle given the difficulty of generating large quantities of actinospores and the extremely high resistance threshold of these fish. For this reason, we chose to focus on two key timepoints in the infection, representing the early and late immune response. We anticipated that these fish would response with a stronger transcriptomic signal due to the higher parasite burden and that the early timepoint would confirm the results of our previous study: that resistant fish mount an earlier immune response to the parasite. Additionally, we believed it would provide a clear view of the early innate immune response, undistorted by the changes in gene expression that arise later in the infection due to the breakdown of the intestinal structure. The late timepoint would help characterize the adaptive immune response, as well as provide a dataset for comparison with susceptible fish from our previous study. We accomplished this by

continuously exposing resistant steelhead to a high concentration of *C. shasta* for 5 days to achieve a high parasite burden in the intestine. Intestines were collected at 7- and 21-dpe and changes in gene expression were analyzed by RNA-seq, and the histopathological response was also investigated at both timepoints.

MATERIALS AND METHODS

Experimental fish

Resistant steelhead were collected from the Round Butte Hatchery (Oregon, USA) and bred as previously described (chapter 2). The fish were reared on 13.5° C specific-pathogen free (SPF) well water at the Oregon State University (OSU) John L. Fryer Aquatic Animal Health Laboratory (AAHL) in Corvallis, Oregon, USA, and fed a commercial diet daily (BioClark's Starter, Bio-Oregon, Longview, Washington, USA).

Parasite challenge

Laboratory cultures of the invertebrate host *Manayunkia occidentalis* are maintained at the AAHL and serve as source of *C. shasta* actinospores for laboratory challenges. The annelids are housed in indoor mesocosms receiving flow-through UV-treated treated river water and spore production is routinely monitored (Hallett and Bartholomew, 2009). The parasite challenge was initiated in March 2019, when spore production from the genotype IIC mesocosm reached 30,000 spores per day, a sufficient dose to meet or exceed their threshold of resistance (Hallett et al., 2012; Ray et al., 2010). 21 resistant steelhead (average 103.1 g ± 7.1 g) were placed in a 100-liter tank that received effluent from the mesocosm. Water temperature was increased from 14.5°C to 17.5°C over 2 days by addition of 18°C SPF well-water. An additional 21 Round Butte steelhead were transferred into an identical tank setup but received effluent from the control mesocosm which contains uninfected *M. occidentalis*. 5 days after the exposure began, the fish (treatment and control) were transferred into six 25-liter tanks (7 fish per tank) that were randomly assigned and supplied with 18°C SPF well-water.

Tissue sampling

The fish were sampled at 7- and 21-days post exposure (dpe), with exposure being defined as their initial placement in the exposure tank. At each timepoint, 3 fish from each of the 6 tanks were euthanized with an overdose of MS-222 (tricaine methanosulfonate, Argent Laboratories, Redmond, WA, USA) for a total of 18 fish per timepoint (9 exposed, 9 controls). The entire lower intestine was removed from each fish and placed in either RNAlater (2 out 3 fish per tank)

or Dietrich's fixative (1 out of 3 fish per tank). The samples collected in RNAlater were immediately stored at 4° C and then transferred to -80° C after 24 hours. At each timepoint, the fish were sampled at the same time of day to avoid changes in gene expression due to circadian rhythms (Reebs, 2002). After the fish were sampled at the 21-dpe timepoint, the remaining fish in each tank were consolidated into two tanks (exposed and control) to eliminate distress due to isolation. The fish were monitored until 60-dpe, at which time they were euthanized with an overdose of MS-222.

Sample processing

The intestine samples collected in RNAlater were homogenized in liquid nitrogen using a porcelain mortar & pestle. 25 mg of homogenized tissue from each sample underwent RNA extraction using the RNeasy Mini Kit (Qiagen, catalog number 74104) according to the manufacture's protocol. An additional 25 mg of tissue underwent DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, catalog number 69506). The extracted DNA was eluted in 30 µl of Buffer AE that was applied to the spin column twice to achieve a higher concentration. The concentration and purity and of the extracted RNA and DNA was assessed with a NanoDrop ND-1000 UV-Vis Spectrophotometer.

The amount of parasite DNA present in the intestine was determined using *C. shasta*-specific qPCR assay (Hallett and Bartholomew, 2009). 100 ng of extracted DNA from each sample was assayed in triplicate wells through 40 cycles using an Applied Biosystems StepOnePlus Real-Time PCR System. A sample was considered positive for *C. shasta* if all three wells fluoresced and the sample was rerun if the Cq standard deviation between wells was greater than 1. On each qPCR plate, a positive control, a negative control (molecular grade water), and a standard dilution curve equivalent to 1, 10, 100, and 1000 actinospores was included.

Intestinal samples in Dietrich's fixative were routinely processed for histology, embedded in Technovit 7100 resin (Heraeus Kulzer, Wehrheim, Germany) and 3 µm sections were stained with Giemsa or with periodic acid Schiff (PAS). Observations and microphotographs were made with a Leitz Dialux 22 light microscope connected to an Olympus DP70 camera.

Library prep and sequencing

Intestinal mRNA from 8 samples at each timepoint (4 treatment, 4 control) were submitted to the Center for Genome Research and Biocomputing at OSU for library preparation and sequencing. The integrity of the RNA was confirmed by running each sample on an Agilent Bioanalyzer 2100 (Agilent Technologies, USA). 1 μg of RNA was used for library preparation using the Illumina TruSeq™ Stranded mRNA LT Sample PrepKit according to the manufacturer's instructions (Cat. No. RS-122-2101, Illumina Inc. San Diego, CA, USA). Library quality was checked with a 4200 TapeStation System (Agilent Technologies, USA) and quantified via qPCR. The libraries were sequenced on two lanes of an Illumina HiSeq 3000 as 100-bp single-end runs.

Data analysis

Adapter sequences were trimmed from the raw reads using BBDuk (January 25, 2018 release) and reads less than 30-bp after trimming were discarded. Library quality was assessed before and after trimming using FastQC (v 0.11.8). The trimmed reads were then mapped to the rainbow trout reference genome (GenBank: MSJN00000000.1) using HiSat2 (v 2.1.0) (Kim et al., 2015). HTSeq-count (v 0.11.1) (Anders et al., 2015) was used to calculated the number of reads that mapped to each gene and the counts were imported into R (v 3.5.0) and loaded into the package DESeq2 (v 1.22.2) (Love et al., 2014). To assess the similarity of the sequenced samples, a PCA plot was constructed using normalized gene counts that were transformed using the rlog function in DESeq2. Hierarchical clustering was performed with the pheatmap package.

Differentially expressed genes (DEGs) between treatment and control fish were identified using the negative binomial Wald test in DESeq2 and were considered significant if they had a Benjamini–Hochberg False Discovery Rate (FDR) adjusted p-value < 0.05 and an absolute $\log_2$(fold change) > 1. Annotation of the DEGs and gene ontology (GO) enrichment was conducted with OmicsBox (v 1.1.135) (Conesa et al., 2005). To obtain high quality annotations, the blast e-value cutoff was set at $1e^{-5}$ and genes were preferentially annotated with the SWISS-PROT database (Bairoch and Apweiler, 2000) followed by the NCBI nonredundant database and the 'Vertebrata' taxonomy filter was applied. All genes detected in this study were used as the background for GO enrichment and up- and downregulated genes were analyzed separately. Enriched GO terms and their FDR-adjusted p-values were imported into Cytoscope (v 3.7.2) (Shannon et al., 2003) for visualization with the ClueGo (v 2.5.6) (Bindea et al., 2009) plugin,

which clusters the GO terms into functionally related networks. *O. mykiss* was chosen as the organism for Ontologies/Pathways and the GO Term Fusion option was used to merge GO terms based on similar associated genes. Volcano plots were constructed with the R package EnhancedVolcano (v 1.0.1) (Blighe et al., 2018).

DEGs found in this study were compared to DEGs previously identified in susceptible steelhead exposed to *C. shasta* (chapter 2). Genes that were differentially expressed in both phenotypes at 21-dpe were identified and the Pearson correlation coefficient between their expression levels was calculated in R. To analyze the antibody response against *C. shasta*, sequenced reads from both resistant and susceptible at 21-dpe were mapped against the coding sequence for the secreted forms of IgM (GenBank: S63348.1) and IgT (GenBank: AY870263.1) using Salmon (v 0.10.0) (Patro et al., 2017). The output was imported into DESeq2 to estimate fold-change between exposed and control fish.

RESULTS

Infection of resistant steelhead

The first clinical sign of *C. shasta* infection occurred at 19-dpe, when all the exposed fish stopped responding to feed, and thus anorexia was evident. At the 21-dpe sampling timepoint, the intestines of all the exposed fish were grossly swollen, with an apparent increase in capillarization and a hemorrhagic appearance. The tissue was also quite stiff and was difficult to cut and later homogenize in liquid nitrogen. Of the three exposed fish that remained after the 21-dpe sampling timepoint, one succumbed to the infection at 32-dpe. Its intestine appeared bloody and swollen and a vent swab revealed abundant mature myxospores. The remaining two fish resumed feeding again at 37-dpe and continued to do so until they were euthanized at 60-dpe. The parasite burden of the exposed fish was quantified by qPCR. The prevalence of infection was 100% among the exposed fish and at 7-dpe they had an average Cq of 27.0 ± 2.0, which increased to 15.2 ± 1.6 at 21 dpe (Fig. 3.1). For reference, the 1 and 1,000 actinospore standards have Cq values of 34 and 24, respectively. All of the control fish were negative by qPCR.

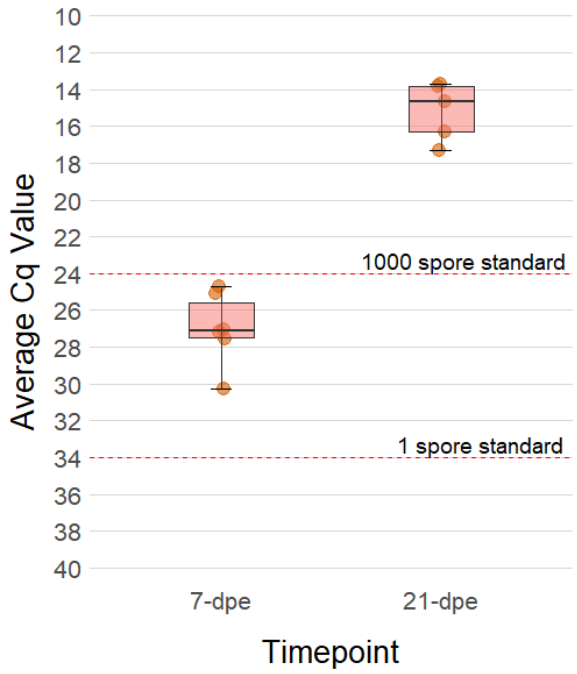

Figure 3.1. Boxplot showing the relative quantity of *Ceratonova shasta* DNA present in the intestines of resistant steelhead at 7- and 21-days post exposure (dpe). Each dot represents the average quantitative cycle (Cq) of 100 ng of DNA extracted from the intestines of one fish that was assayed in triplicate by qPCR. Six fish were assayed at each timepoint. Dashed red lines indicate the average Cq values obtained from 1 and 1000 actinospore standards.

Histopathology

Histological observation found a substantial hyperplasia in the *lamina propria*-submucosa of the infected fish at 21-dpe, which was not observed in control fish (Fig. 3.2d). At 7-dpe, intestines of infected fish maintained their basic tissue structure, though cell proliferation and infiltration in the submucosa was patent. These signs were significantly magnified in the intestines of infected fish at 21-dpe, which presented severe submucosal hyperplasia. At this timepoint, trophozoites as well as disporoblasts with mature spores were observed invading the aberrant submucosa, but they did not reach beyond the *stratum compactum* (Fig. 3.3b). This collagen sheath, which lies beneath the *lamina propria*, seemed to act as a barrier, preventing parasite from spreading into deeper layers. Inflammatory cell foci in infected fish mainly consisted of lymphocyte-like cells, and some granulocytes, that were present in the submucosa and infiltrating the epithelium.

Interestingly, the fish that survived until 60-dpe were able to largely regenerate their intestinal structure. Large clusters of basophils were present along the submucosa (Fig. 3.3). However, the parasite was not completed cleared by 60-dpe, and *C. shasta* stages were still found, mainly in the intestinal lumen (Fig. 3.2).

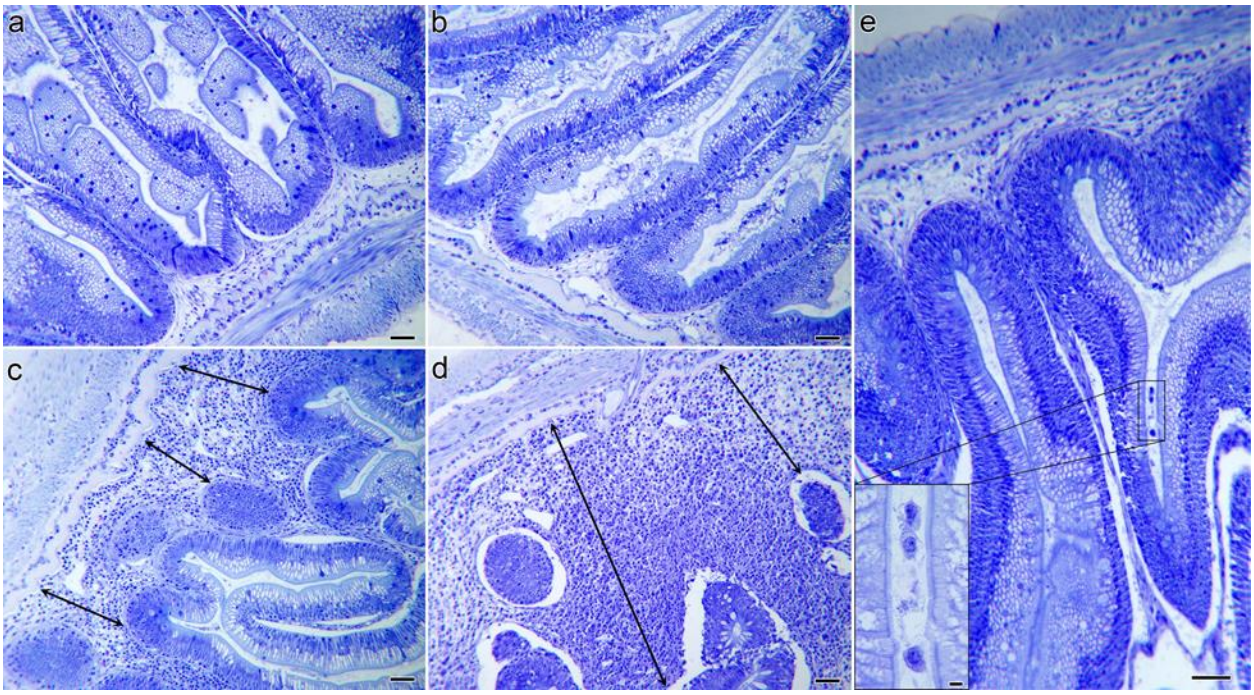

Figure 3.2. Histopathology of resistant steelhead. Intestinal structure of unexposed control (a, b) and infected (c, d, e) resistant steelhead at 7 days post exposure to Ceratonova shasta (a, c), at 21 days (b, d) and 60 days (e). Note the mild (c) and severe (d) submucosal hyperplasia in recipient fish at the two early time points (double arrowheads). At 60 days post exposure, the intestinal tissue structure has recovered and resembles that of controls (e). Note the presence of parasite stages in the intestinal lumen (insert). All slides are stained with Giemsa. Scale bars = 50 µm, except in insert = 5 µm.

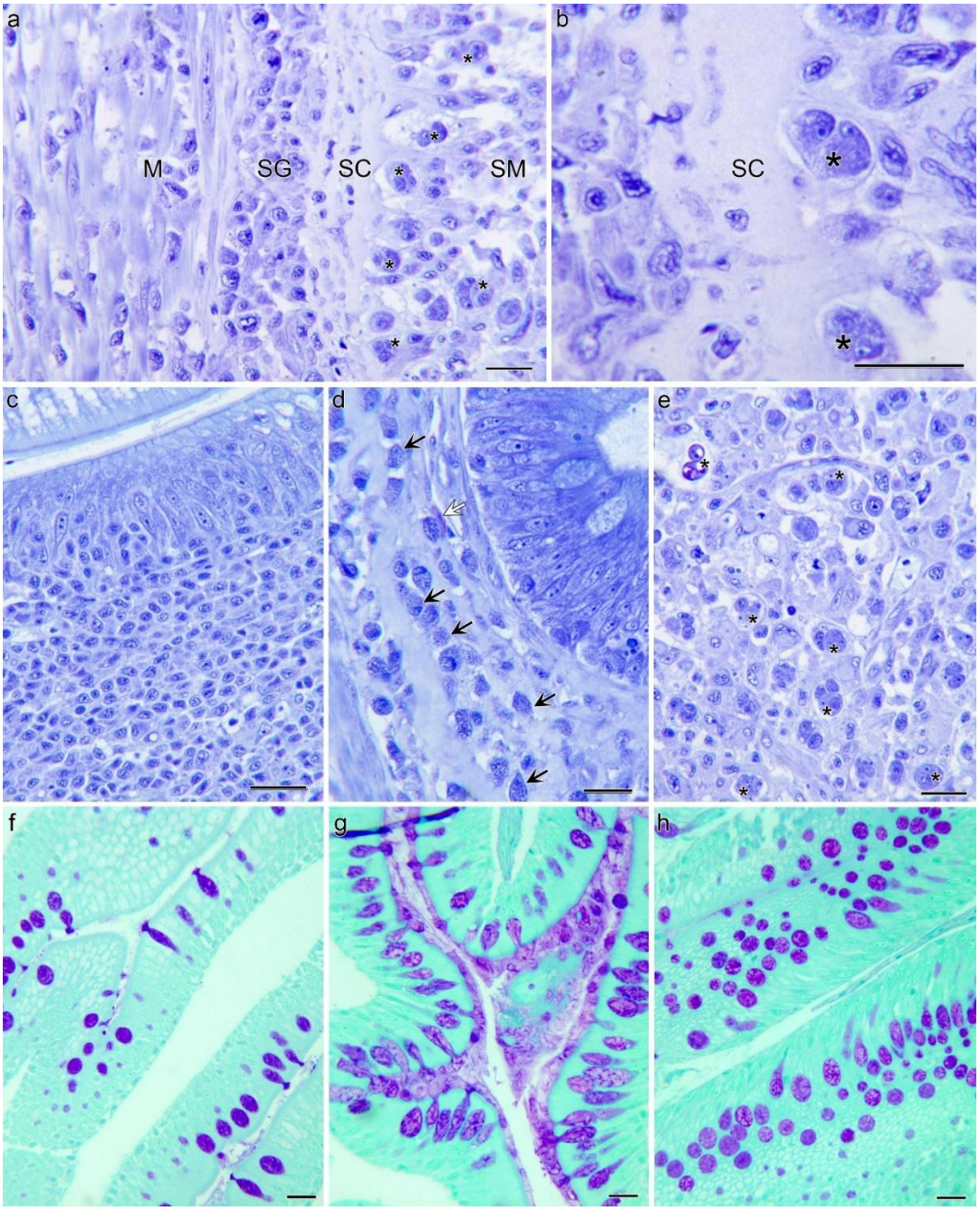

Figure 3.3. Intestinal histopathology of resistant steelhead exposed to *Ceratonova shasta*. At 21 days post exposure (dpe) (a, b, e) parasite stages (asterisks) present in the intestinal submucosa (SM) reach the stratum compactum (SC) but do not invade the underlying stratum granulosum (SG) and muscularis (M). Note that the lamina propria-submucosa is invaded by the parasite and has lost its tissue structure (e). Inflammatory cell proliferation and infiltration at 7-dpe consisting of lymphoblast-like cells (c). Cluster of granulocytes (black arrows) in the submucosa at a 60-dpe (d). Note the scarce presence of eosinophils (white arrow). Mucins stained with periodic acid Schiff (PAS) (magenta) in 7-dpe (f), 21-dpe (g) and 60-dpe (h) intestines. Note the high abundance of PAS+ goblet cells at the two latter time points. Images (a-e) = Giemsa, images (f-h) = PAS+. Scale bars = 20 µm.

Sequencing of intestinal mRNA

The sequencing of intestinal mRNA from resistant fish at 7- and 21-dpe generated 519 million single-end reads. The percentage of reads that could be uniquely mapped to a single locus in the rainbow trout genome varied between groups, ranging from 40.4% to 75.4% (Table 3.1). Principle component analysis showed a high degree of uniformity between samples within a group and clearly separated infected fish from controls with the first principle component explaining 63% of the total variation (Fig. 3.4). Hierarchical clustering also clearly separated the infected samples from 21-dpe (Fig. 3.5) and all the samples showed a high degree of correlation ($r > 0.96$), suggesting no outliers were present.

Table 3.1. Sequencing and mapping results.

Group	Average number of reads	Average number of mapped reads
Controls - 7 dpe	29,536,649	21,403,620 (72.5%)
Infected - 7 dpe	24,588,191	17,768,446 (72.2%)
Controls - 21 dpe	44,080,197	33,526,343 (75.4%)
Infected - 21 dpe	31,730,221	11,964,621 (40.4%)

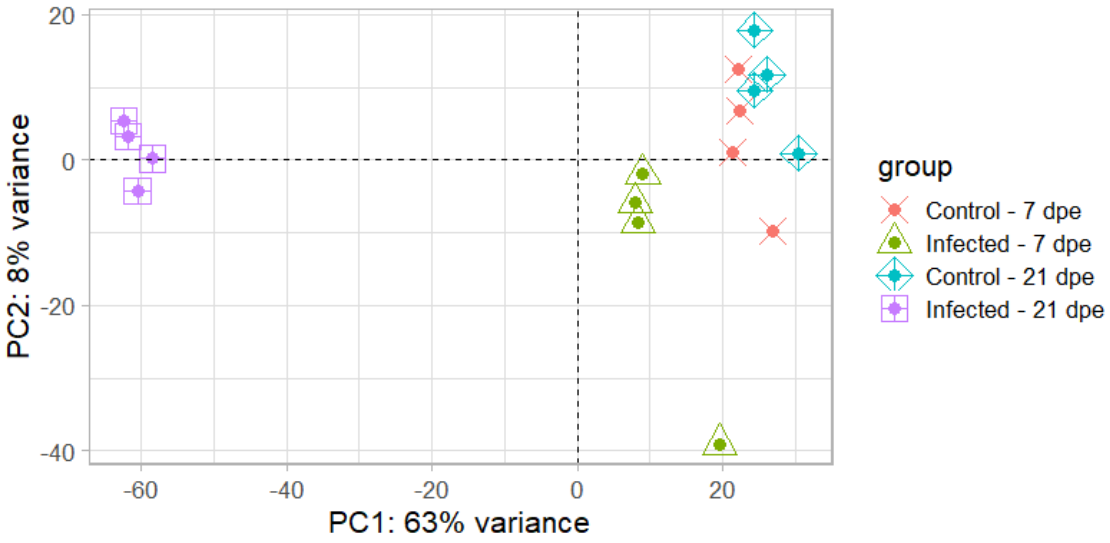

Figure 3.4. Principle component analysis of the regularized log transformed gene counts for each sample

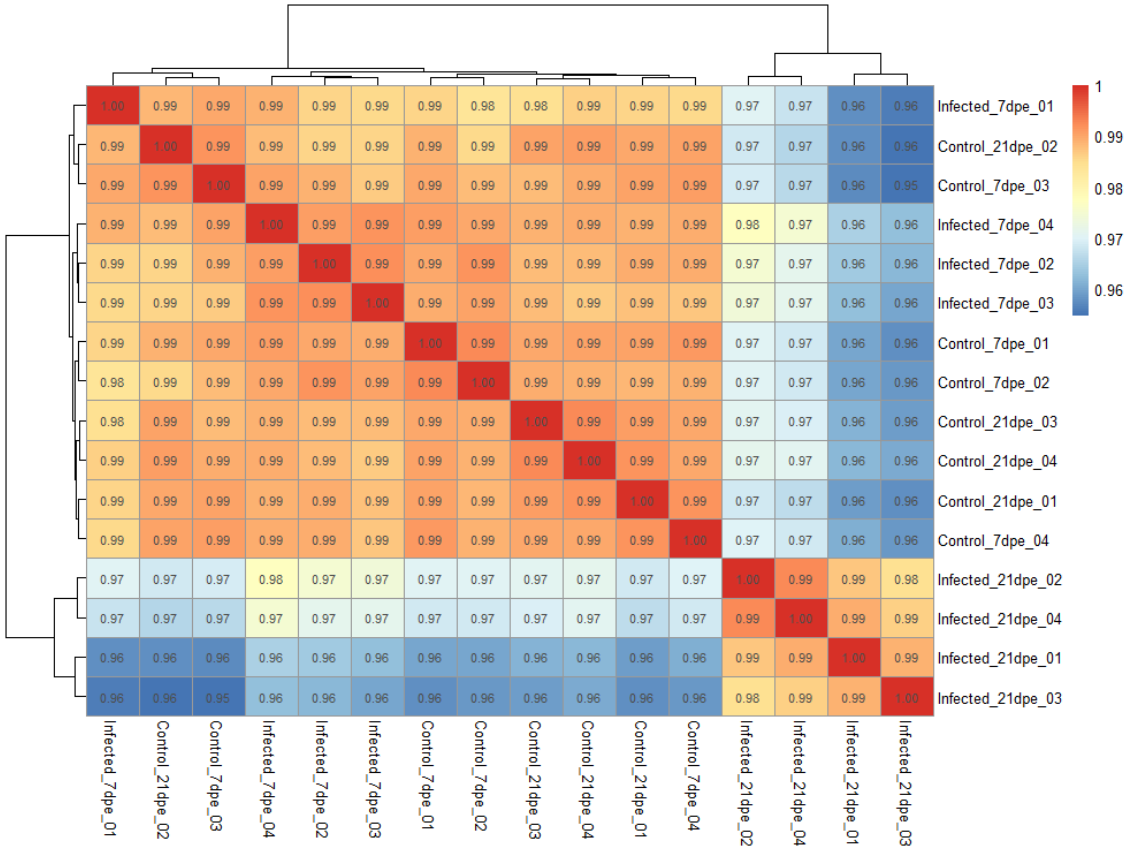

Figure 3.5. Hierarchical clustering and heatmap showing the Pearson correlation coefficient (r) between each sample. Values were generated from rlog transformed gene counts for each sample using the R packages DESeq2 and Pheatmap.

Differential gene expression in the intestine at 7-dpe and analysis of key genes

The sequencing reads from 7-dpe were mapped to 32,959 genes in the rainbow trout genome, and 417 differentially expressed genes (DEGs) were identified, including 338 (81%) upregulated genes and 78 (19%) downregulated genes (Fig. 3.6). Among the DEGs, 30 genes were strongly upregulated (Log2-FC > 4), including C-C motif chemokine 4-like (285.6 fold), two copies of C-C motif chemokine 13-like (46.7, 79.9 fold), three copies of C-C motif chemokine 19-like (27.8, 42.7, 51.4 fold), two copies of caspase-14-like (34.7, 139.2 fold), five copies of interferon-induced protein 44-like (20.6, 42.8, 68.7, 82.8, 86.4, 161.2 fold), interferon-gamma 2 precursor (32.4 fold), and cathelicidin 1 precursor (38.1 fold). Five genes were found to be strongly downregulated: protein CREG1-like (-969.7 fold), troponin T - fast skeletal muscle isoforms (-78.5 fold), cytochrome P450 2K4 (-38.4 fold), C-X-C motif chemokine 13-

like (-29.2 fold), CUGBP Elav-like family member 3-B (-23.8 fold), and guanylyl cyclase-activating protein 2-like (-19.6 fold).

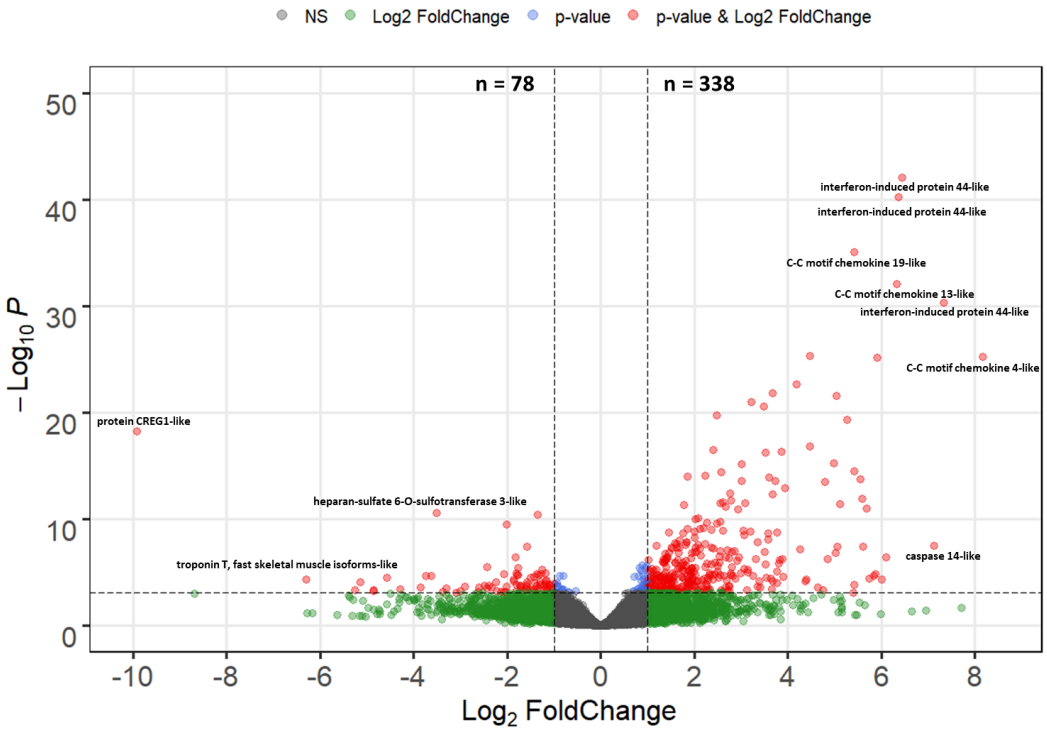

Figure 3.6. Volcano plot of the differential gene expression in the intestine of resistant steelhead at 7-days post exposure to *Ceratonova shasta*. Each dot represents the average value of one gene across four biological replicates. Red indicates the gene was significant at the FDR-adjusted p-value and Log2-Foldchange threshold, blue is significantly only by p-value, green only by Log2-FoldChange, and gray were not significant by either metric.

Based on the DEGs functional description and their associated GO terms, 114 DEGs were grouped into ten categories and presented in table 3.2.

Table 3.2. Selected genes that were differentially expressed in the intestine of resistant steelhead at 7-days post exposure to *Ceratonova shasta*.

Entrez Gene ID	Protein Product	Fold
Cell adhesion and Migration		
LOC110493534	beta-parvin-like	2.2
LOC110530422	cadherin-2-like	2.5

LOC110488853	CD97 antigen-like	6.3
LOC110537995	cell adhesion molecule 4-like	3.7
LOC110534540	claudin-1-like	4.0
LOC110524880	E-selectin-like	3.8
LOC110531988	fibulin-2-like	6.4
LOC110529577	gap junction Cx32.2 protein-like	2.7
LOC110495444	gap junction gamma-1 protein-like	-4.1
LOC110537004	integrin alpha-2-like	3.6
LOC100653476	integrin alpha-4	2.9
LOC110528502	integrin alpha-5-like	3.8
LOC110509064	peroxidasin-like protein	13.5
LOC110486368	vascular cell adhesion protein 1-like	2.9
Antigen Processing and		
LOC110488346	proteasome subunit beta type-7-like	2.1
LOC110488347	proteasome subunit beta type-8-like	2.5
LOC110488348	proteasome subunit beta type-9	2.6
LOC110528193	E3 ubiquitin-protein ligase DTX3L-like	2.2
LOC110523717	E3 ubiquitin-protein ligase NEURL3-like	31.7
LOC110487518	E3 ubiquitin-protein ligase RNF135-like isoform X3	14.1
LOC110537403	E3 ubiquitin-protein ligase RNF144A-like	2.6
LOC110485543	E3 ubiquitin-protein ligase RNF144B-like	2.8
LOC110524942	E3 ubiquitin-protein ligase rnf213-alpha-like	5.9
LOC110531504	E3 ubiquitin-protein ligase TRIM39-like	9.3
LOC110497030	E3 ubiquitin-protein ligase TRIM69-like	12.5
LOC110534395	probable E3 ubiquitin-protein ligase HERC3	7.9
LOC110500498	probable E3 ubiquitin-protein ligase RNF144A-A	5.2
LOC110500501	ubiquitin carboxyl-terminal hydrolase 47-like	3.1
LOC110536452	ubiquitin-conjugating enzyme E2 L3-like	2.1
LOC110530687	ubiquitin-conjugating enzyme E2 R1-like	2.0
LOC110494671	ubiquitin-like modifier-activating enzyme 1	3.4
LOC100136299	tapasin long form precursor	5.8
LOC100136300	tapasin-related	3.4
LOC110500638	tapasin-related protein-like	4.0
tap2a	TAP2a protein	2.1
LOC110535123	endoplasmic reticulum aminopeptidase 1-like	3.4
LOC110526886	endoplasmic reticulum aminopeptidase 2-like	3.0
LOC110487866	antigen peptide transporter 1-like	3.2
LOC110488345	antigen peptide transporter 2-like	6.1
ly75	lymphocyte antigen 75 precursor	3.2
LOC110504514	lymphocyte function-associated antigen 3-like	2.0
LOC110514021	CD83 antigen-like	8.3
LOC110501369	major histocompatibility complex class I-related gene	3.4
LOC110506874	major histocompatibility complex class I-related gene	5.8
Chemokines		
LOC110512719	C-C chemokine receptor type 5-like	5.0
LOC110530382	C-C chemokine receptor type 8-like	2.7
LOC100135979	CC chemokine with stalk CK2 precursor	4.9
LOC110514657	C-C motif chemokine 13-like	79.9
LOC110536449	C-C motif chemokine 19-like	51.4
LOC110536450	C-C motif chemokine 4-like	285.6
cxcf1a	chemokine CXCF1a precursor	4.7
cxcf1b	chemokine CXCF1b precursor	3.8
cxcl13	chemokine CXCL13 precursor	13.6
LOC100136107	chemokine receptor-like protein 1	4.2
LOC110509178	chemokine XC receptor 1-like	2.5
LOC110485791	C-X-C motif chemokine 11-like	12.7
ccl13	Small inducible cytokine A13 precursor	33.0
Interferon Stimulated Genes		
irf-1	interferon regulatory factor 1	11.4

LOC110533376	interferon regulatory factor 1-like	2.3
LOC110492403	interferon regulatory factor 4-like	5.6
LOC110526480	interferon regulatory factor 8-like	3.5
LOC110538600	interferon-induced 35 kDa protein homolog	2.3
mx	interferon-induced GTP-binding protein Mx1	5.2
LOC110520643	interferon-induced GTP-binding protein Mx-like	13.9
LOC110494016	interferon-induced protein 44-like	161.2
LOC110487764	stimulator of interferon genes protein-like	4.9
Pattern Recognition Receptors		
LOC110489499	C-type lectin domain family 4 member E-like	2.7
LOC110517676	C-type lectin domain family 9 member A-like	11.5
LOC110508265	macrophage mannose receptor 1-like	12.8
LOC110496441	toll-like receptor 13	12.6
LOC110507636	high affinity immunoglobulin gamma Fc receptor I-like	3.3
LOC110523269	protein NLRC5	3.9
LOC110531495	fucolectin-4	2.3
Cytokines		
socs1	suppressor of cytokine signaling 1	4.0
LOC110495002	suppressor of cytokine signaling 1-like	12.0
LOC110512513	suppressor of cytokine signaling 3-like	2.9
LOC110520020	signal transducer and activator of transcription 1-	2.4
ifngamma2	interferon gamma 2 precursor	32.4
LOC100135968	interleukin 13 receptor alpha-2	5.9
il12b	interleukin-12 beta chain precursor	5.3
LOC110500135	interleukin-15 receptor subunit alpha-like	2.2
il2rc2	interleukin-2 receptor gamma chain-2 precursor	3.7
Adaptive immune response		
LOC101268951	tumor necrosis factor receptor superfamily member 5	4.0
tnfrsf5a	TNF receptor superfamily member 5A precursor	2.7
LOC110497707	TNF receptor-associated factor 3-like	2.9
LOC110496128	BLIMP-1	3.6
LOC110537828	B-cell antigen receptor complex-associated protein alpha	4.4
LOC110537869	B-cell lymphoma 3 protein homolog	2.6
LOC110516594	B-cell receptor CD22-like	6.7
LOC110498810	leukocyte antigen CD37-like	4.0
LOC110485442	GTPase IMAP family member 4-like	3.8
LOC110533001	GTPase IMAP family member 7-like	12.0
Proteases and Protease		
LOC110521419	mast cell protease 2-like	8.4
LOC110530599	matrix metalloproteinase-14-like	3.4
LOC110494201	matrix metalloproteinase-19-like	3.9
LOC110491324	matrix metalloproteinase-25-like	6.7
LOC110538774	leukocyte elastase inhibitor-like	2.1
LOC110538848	metalloproteinase inhibitor 2-like	2.2
Apoptosis		
LOC110536971	programmed cell death 1 ligand 1-like	3.8
LOC110495001	programmed cell death protein 4-like	48.8
LOC110537938	apoptosis regulator BAX-like	2.3
LOC110491862	tumor necrosis factor receptor superfamily member 6B-like	8.4
LOC110503799	caspase-1-like	2.9
LOC100135902	caspase 6 precursor	3.2
LOC110523645	caspase recruitment domain-containing protein 8-like	4.8
LOC110492801	calpain-1 catalytic subunit-like	2.4
LOC110534030	calpain-5-like	5.7
Others		
LOC110511404	caspase-14-like	139.2
LOC110511405	caspase-14-like	34.7
LOC110506002	arginase-2, mitochondrial-like	4.8
e7	type I keratin E7	5.0

LOC100136204	cathelicidin 1 precursor	38.1
LOC110523368	pyrin-like	6.8
LOC100135935	hepcidin	28.6

GO enrichment 7-dpe

GO enrichment analysis was conducted on the upregulated genes to gain insight into the biological functions of these DEGs, as well as the involved molecular processes. This identified 115 significantly enriched GO terms among the upregulated genes. ClueGo analysis clustered these terms into networks revolving around GO terms for “defense response to other organism”, “defense response”, “innate immune response”, and “interferon gamma-mediated signaling pathway” (Fig. 3.7). No specific GO enrichment was found among the downregulated genes.

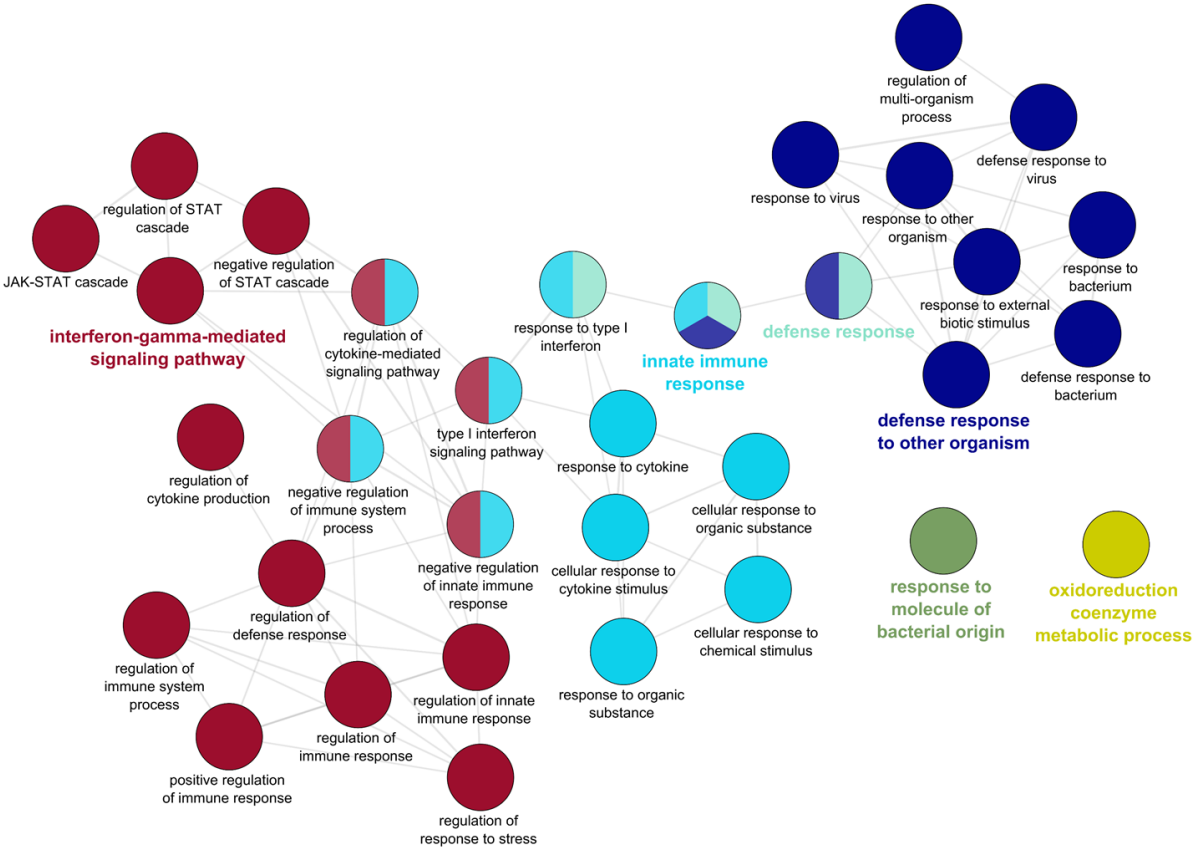

Figure 3.7. Gene ontology (GO) enrichment of the genes upregulated in the intestine of resistant steelhead at 7-days post exposure to *Ceratonova shasta*. Enriched GO terms were grouped into functionally related nodes using ClueGO, a Cytoscape plugin. Nodes are colored and grouped according to related functions and labelled by the most significant term of the group. Node size corresponds to the FDR-adjusted p-value of each GO term and is specific to each graph.

Differential gene expression in the intestine at 21-dpe and analysis of key genes

The sequencing reads from 21-dpe mapped to 34,286 genes and 11,984 DEGs were identified. Of these, 5,801 (48%) were upregulated and 6,183 (52%) were downregulated. In addition to the increased number of DEGs at 21-dpe, the magnitude of the differential expression also greatly increased, with 1,149 DEGs being strongly upregulated (Log2-FC > 4) (Fig. 3.8).

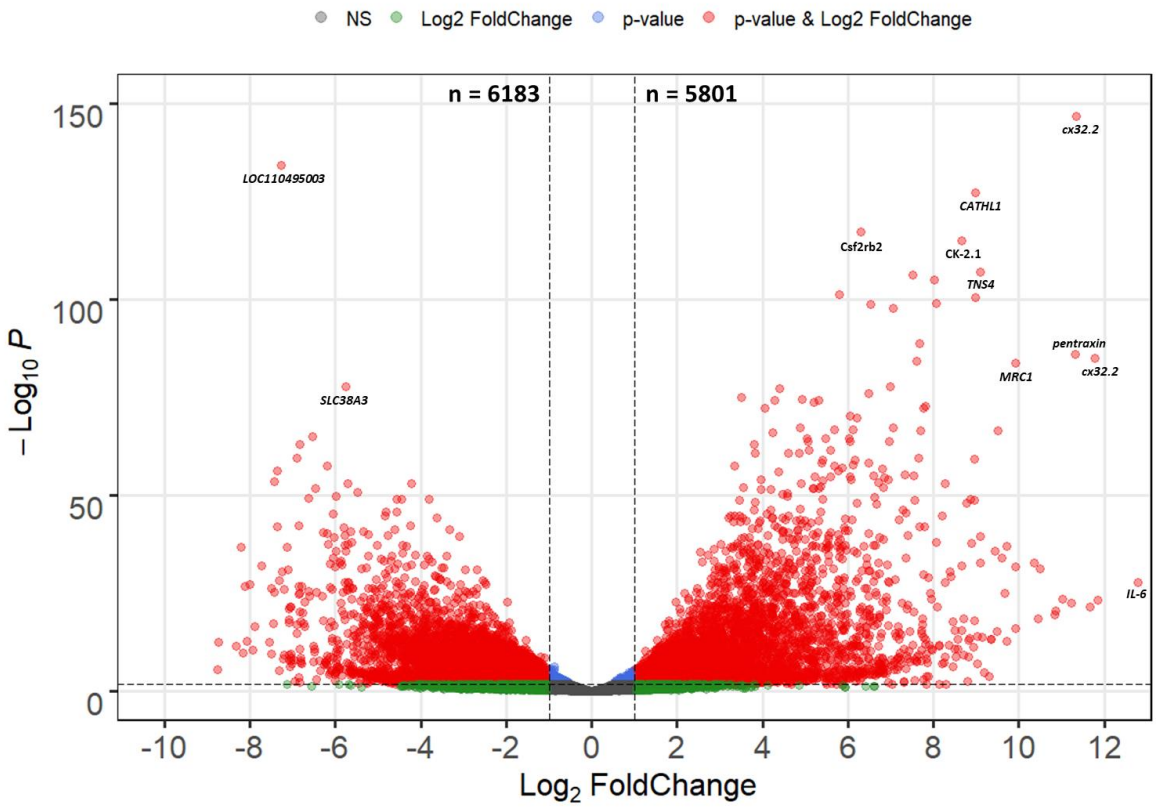

Figure 3.8. Volcano plot of the differential gene expression in the intestine of resistant steelhead at 21-days post exposure to *Ceratonova shasta*. Each dot represents the average value of one gene across four biological replicates. Red indicates the gene was significant at the FDR-adjusted p-value and Log2-Foldchange threshold, blue is significantly only by p-value, green only by Log2-FoldChange, and gray were not significant by either metric.

The most highly upregulated genes were those related to the immune system, cell communication, and tissue remodeling: interleukin-6-like (7118.6 fold), two copies of gap junction Cx32.2 protein-like (3516.0 and 2584.8 fold), chemokine CXCL13 precursor (3258.9 fold), interleukin-6 precursor (1851.8 fold), interleukin-10 precursor (1393.7 fold), fibroblast growth factor 21 (967.4 fold) and interleukin-1 beta-like (965.1 fold). The most significantly

downregulated genes were primarily involved in metabolic pathways and transport of ions and solutes, as well as a few immune genes: solute carrier family 13 member 3-like (-430.3 fold), beta,beta-carotene 9',10'-oxygenase-like (-323.4 fold), complement factor B (-276.2 fold), aquaporin-8-like (-176.4 fold), interferon-induced GTP-binding protein Mx2-like (-171.6 fold), and gastrotropin-like (-163.7 fold).

GO enrichment 21-dpe

GO analysis of the upregulated genes revealed that they were enriched for 1,135 GO terms, predominantly immune-related. ClueGO analysis clustered the biological process GO terms into five networks revolving around the terms “transmembrane receptor protein tyrosine kinase signaling pathway”, “immune response-activating signal transduction”, “cytokine secretion”, “immune response-activating cell surface receptor signaling pathway”, “T cell differentiation”, regulation of leukocyte migration”, and “positive regulation of actin filament polymerization” (Fig. 3.9).

Figure 3.9. Gene ontology (GO) enrichment of the genes upregulated in the intestine of resistant steelhead at 21-days post exposure to *Ceratonova shasta*. Enriched GO terms were grouped into functionally related nodes using ClueGO, a Cytoscope plugin. Nodes are colored and grouped according to related functions and labelled by the most significant term of the group. Node size corresponds to the FDR-adjusted p-value of each GO term and is specific to each graph. Continued on next page.

The same analysis of the downregulated genes found 491 enriched GO terms which ClueGO analysis clustered into networks based on metabolic and energy producing GO terms, including “generation of precursor metabolites and energy”, and “carboxylic acid catabolic process” (Fig. 3.10).

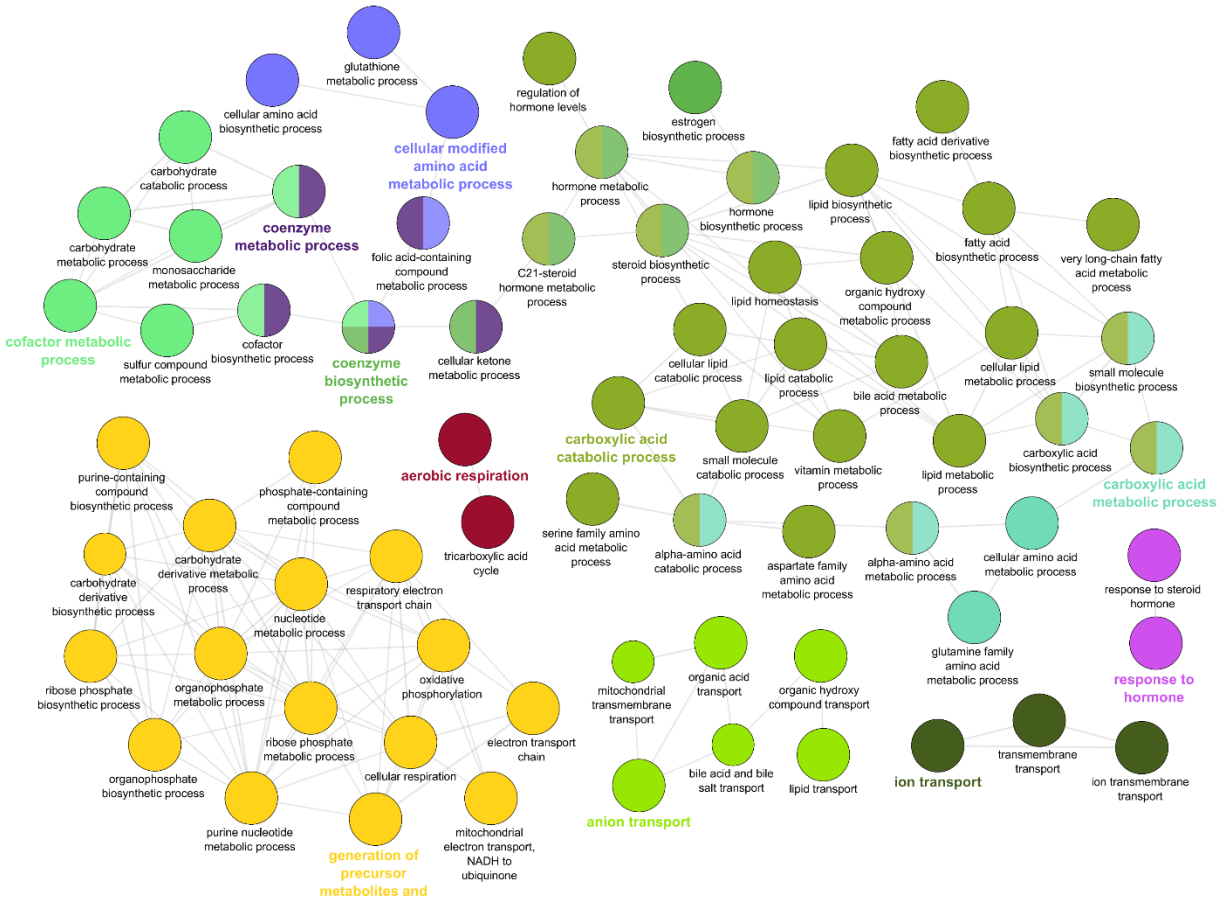

Figure 3.10. Gene ontology (GO) enrichment of the genes downregulated in the intestine of resistant steelhead at 21-days post exposure to *Ceratonova shasta*. Enriched GO terms were grouped into functionally related nodes using ClueGO, a Cytoscape plugin. Nodes are colored and grouped according to related functions and labelled by the most significant term of the group. Node size corresponds to the FDR-adjusted p-value of each GO term and is specific to each graph.

Analysis of key immune genes

Based on the GO enrichment profiles and the most highly upregulated genes, a set of 89 key genes related to the innate and adaptive immune response were identified and are presented in table 3.3.

Table 3.3. Selected immune genes that were differentially expressed in the intestine of resistant steelhead at 21-days post exposure to *Ceratonova shasta*.

Entrez Gene ID	**Protein Product**	**Fold Change**
T-cells		
cd3z	T-cell surface glycoprotein CD3 zeta chain	8.0
LOC100136285	T-cell surface glycoprotein CD4	5.6
onmy-cd8a	T-cell surface glycoprotein CD8 alpha precursor	-9.2
LOC100136222	CD8 beta precursor	-6.1
LOC110495954	T-cell-specific surface glycoprotein CD28-like	9.8
LOC110500642	CD276 antigen-like	217.3
LOC100136275	CTLA4-like protein precursor	44.7
LOC110509520	lymphocyte activation gene 3 protein-like	377.5
prkcq	protein kinase C theta type	12.5
LOC110536971	programmed cell death 1 ligand 1-like	65.4
LOC110489621	V-set domain-containing T-cell activation inhibitor 1-like	-10.1
LOC110516762	GTPase IMAP family member 7-like	38.9
T_H1		
ifng	interferon gamma precursor	90.9
ifngamma2	interferon gamma 2 precursor	249.0
ifngr1	interferon gamma receptor 1	16.1
ifngr1	interferon-gamma receptor alpha chain precursor	3.7
irf-1	interferon regulatory factor 1	6.2
LOC110506608	interferon regulatory factor 8-like	8.6
il12b	interleukin-12 beta chain precursor	89.2

LOC110524480	interleukin-12 receptor subunit beta-2-like	-3.1
LOC110524481	interleukin-12 receptor subunit beta-2-like	9.8
LOC110508876	interleukin-12 subunit alpha-like	47.2
LOC110489482	interleukin-12 subunit beta-like	-4.2
LOC110511354	interleukin-18 receptor accessory protein-like	20.2
LOC110501544	signal transducer and activator of transcription 1-alpha/beta-like	6.4
tbx21	T-box 21	33.0
T_H2		
il4/13a	interleukin-4/13A precursor	52.9
LOC110489171	interleukin-4/13b1 precursor	72.8
LOC110504551	interleukin-4/13b2 precursor	774.4
LOC110500122	transcription factor GATA-3-like	6.1
stat5	signal transducer and activator of transcription 5	7.7
socs3	suppressor of cytokine signaling 3	46.4
T_H17		
LOC110529296	interleukin-17A-like	25.6
LOC110524663	interferon regulatory factor 4-like	31.5
LOC110520784	nuclear receptor ROR-gamma-like	-22.6
LOC110535950	nuclear receptor ROR-gamma-like	-16.0
T-reg		
il10	interleukin-10 precursor	1393.7
LOC100136774	transforming growth factor beta-1	3.9
Cytotoxic and NK cells		
LOC110538116	perforin-1-like	106.3
LOC110536463	granzyme A-like	10.3
LOC110520655	granzyme B-like	7.7
LOC110524258	granzyme-like protein 2	7.2
LOC110500840	natural killer cell receptor 2B4-like	95.1
LOC110498133	antimicrobial peptide NK-lysin-like	31.8
B-cells		

LOC110522002	BLIMP-1	223.7
LOC110496128	BLIMP-1	55.1
LOC110499048	tumor necrosis factor receptor superfamily member 13B-like	1821.4
LOC110491449	tumor necrosis factor receptor superfamily member 13B-like	7.8
LOC110494997	B-cell receptor CD22-like	37.7
LOC110537828	B-cell antigen receptor complex-associated protein alpha chain-like	24.3
LOC110537869	B-cell lymphoma 3 protein homolog	19.5
LOC110521598	polymeric immunoglobulin receptor-like	69.7
nilt2	polymeric immunoglobulin receptor-like precursor	-3.4
cd79b	CD79b	5.4
klhl6	kelch-like protein 6	16.5
Macrophages		
LOC110520098	interleukin-34-like	6.2
csf1	macrophage colony-stimulating factor precursor	4.0
LOC110508265	macrophage mannose receptor 1-like	978.1
LOC110508267	macrophage mannose receptor 1-like	224.3
LOC110536912	macrophage mannose receptor 1-like	-159.8
LOC100136664	macrophage myristoylated alanine-rich C kinase-like protein	34.6
LOC110520391	macrophage receptor MARCO-like	4.3
LOC100136179	arginase-1	36.5
LOC110498289	arginase-2, mitochondrial-like	42.6
Granulocytes		
csf-3	granulocyte colony-stimulating factor precursor	143.1
LOC100136240	neutrophil cytosolic factor 2	21.7
LOC110523686	eosinophil peroxidase-like	108.4
LOC110524274	mast cell protease 1A-like	14.6
Cytokines		
LOC110536401	interleukin-1 beta-like	965.1
il-6	interleukin-6 precursor	1851.8

LOC110496949	interleukin-6-like	7118.8
il-8	putative CXCL8/interleukin-8	476.2
socs1	suppressor of cytokine signaling 1	9.6
LOC110532426	suppressor of cytokine signaling 2-like	-7.2
socs5	suppressor of cytokine signaling 5	2.3
tnf	tumor necrosis factor	12.2
Chemokines		
cxcl13	chemokine CXCL13 precursor	3258.9
LOC110490829	C-C chemokine receptor type 3-like	1443.9
LOC110494122	C-C motif chemokine 4 homolog	1313.9
LOC100135979	CC chemokine with stalk CK2 precursor	403.6
LOC110514657	C-C motif chemokine 13-like	232.9
LOC110485791	C-X-C motif chemokine 11-like	173.5
LOC110525193	C-C motif chemokine 19-like	83.1
Other		
LOC100653444	hepcidin-like	2399.5
LOC110490701	complement C1q tumor necrosis factor-related protein 3-like	2098.0
LOC100136204	cathelicidin 1 precursor	502.4
LOC100136187	cathelicidin antimicrobial peptide	234.4
LOC110514021	CD83 antigen-like	15.0
LOC110533206	C-type lectin domain family 4 member M-like	30.2

Comparison to the transcriptome of susceptible fish at 21-dpe

In order to understand what is the difference between the response of resistant fish and their susceptible counterparts, we compared the differential gene expression results from 21-dpe in this study to those previously generated for susceptible fish with a similar parasite burden at 21-dpe (chapter 2). Comparison of the 11,984 DEGs identified in resistant fish at 21-dpe with the 12,061 DEGs found in susceptible fish at 21-dpe identified 7,820 genes differentially expressed in both phenotypes (Fig. 3.11). 28 of the 7,820 were expressed in opposite directions in resistant fish compared to susceptible fish (supplemental). Among the 28 genes, 2 copies of “60 kDa heat

shock protein, mitochondrial" were the only genes with known immune functions and were downregulated in resistant fish (-2.3 & -2.5-Fold). The overall expression of the shared DEGs was highly positively correlated ($r(7820) = 0.94$, $p < 2.2e\text{-}16$), and the average difference in fold change was 1.76 ± 1.73. GO enrichment analysis of the shared DEGs revealed 453 enriched GO terms, primarily related to metabolic processes, innate immune response, and cell adhesion. Analysis of the genes that were differentially expressed only in resistant fish revealed a similar profile with 302 enriched GO terms. However, adaptive immune system processes were much more prominent, including many terms that were not present among the shared DEGs or those unique to susceptible fish. These included "adaptive immune response", "adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains", "lymphocyte mediated immunity", and "T cell mediated immunity."

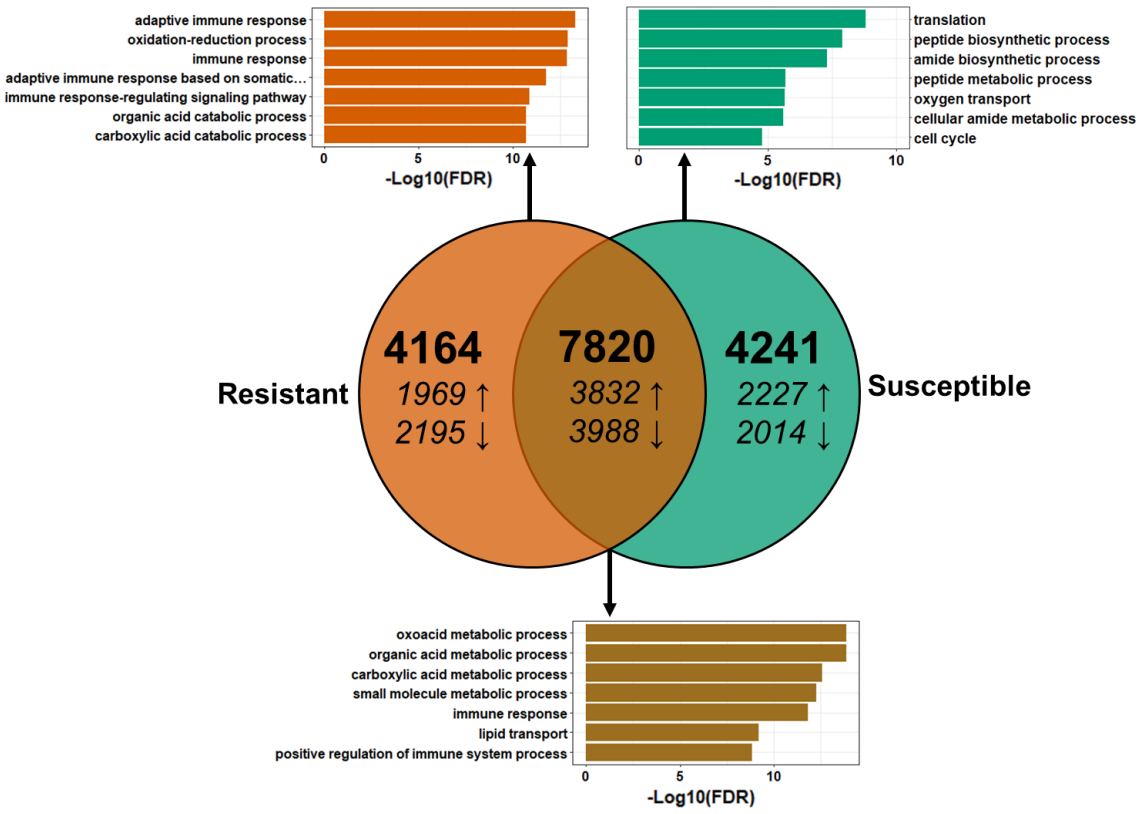

Figure 3.11. Comparison of the differential gene expression between resistant and susceptible steelhead at 21-days post exposure to *Ceratonova shasta*. Venn diagram showing the number of differentially expressed genes (DEGs) shared between resistant and susceptible steelhead. Arrows indicate upregulation vs. downregulation. The top seven most statistically significant enriched GO terms for each set of DEGs are shown in the corresponding bar graphs.

The number of secreted IgT and IgM transcripts was quantified for both resistant and susceptible fish at 21-dpe to compare the antibody response between the two phenotypes. This revealed a much stronger induction of both immunoglobulins in resistant fish, particularly IgT, which was upregulated over 300-fold (table 3.4).

Table 3.4. Comparison of the fold change of secreted IgT and IgM transcripts in the intestine of resistant and susceptible steelhead at 21-days post exposure to *Ceratonova shasta*

	Resistant		**Susceptible**	
	Fold change	(FDR) p-value	Fold	(FDR) p-value
IgT	336.9	1.03E-24	65.5	2.27E-08
IgM	14.5	1.03E-17	2.3	0.219763

DISCUSSION

The dialogue between host and parasite is complex, involving not only the immune system, but changes in the host's metabolism and the cellular structure of the infected tissues. RNA-seq offers a broad, non-targeted approach to understanding this process, and can identify putative resistance genes for future functional studies. *C. shasta* offers an attractive model for studying the host-parasite dynamic, given that it infects well-studied and economically important salmonids that naturally occur on the phenotypic extremes of resistance. In this study, we sequenced intestinal RNA from resistant steelhead that were exposed to a high dose of *C. shasta* to elicit a vigorous immune response. Samples from 7- and 21-dpe were sequenced to further our understanding of the innate and adaptive immune response to this parasite. We found that by 7-dpe resistant fish were already mounting a vigorous IFN-γ driven T_H1 response that was accompanied by remodeling of the intestinal tissue. This continued at 21-dpe, where signs of a strong antibody response were evident along with a possible transition to a T_H2 response. Despite the overwhelming parasite dose these fish received and the damage to the intestinal structure that resulted, some fish were able to regenerate their intestinal tissue and resume feeding as normal.

In our previous study comparing the transcriptomic response of resistant and susceptible steelhead exposed to a moderate dose of *C. shasta* (chapter 2), we observed that despite having a significant parasite burden in their intestine by 7-dpe, susceptible fish did not respond to the infection. Although the exposure was not sufficient to establish an active infection in the

intestines of resistant fish, we observed upregulation of several immune genes in the intestine at 7-dpe, suggesting a more rapid immune response was occurring in resistant fish. The results presented here confirm this, with resistant fish mounting a vigorous immune response at 7-dpe, with 338 genes upregulated. This response was predominantly an IFN-γ driven T_H1 response, with upregulation of numerous interferon stimulated genes, as well as MHC class I genes and those involved in antigen processing and presentation. This would suggest that an adaptive immune response is already being mounted against the parasite. We also observed upregulation of NLRC5 and GTPase IMAP family member 4 (GIMAP 4). NLRC5 is a cytosolic pathogen recognition receptor involved in MHC class I-dependent immune responses (Benkő et al., 2017) and was found to be upregulated in the gills and intestine of resistant fish in our previous study. In that same study, two paralogs of GIMAP 4, a protein involved in T-lymphocyte development (Filén and Lahesmaa, 2010), were the most highly upregulated genes in the gills of resistant fish at 1-dpe. Intriguingly, the exact same paralogs were the most highly upregulated immune genes in the intestine of susceptible fish at 14-dpe. The early induction of these genes in resistant fish compared to susceptible fish strongly suggests a role for these genes in *C. shasta* resistance. Although different paralogs of NLRC5 and GIMAP 4 were identified in this study, our findings further support their involvement in *C. shasta* resistance.

It is also important to highlight that despite being exposed to much higher concentration of *C. shasta*, and over a longer period of time, 2-fold less parasite DNA could be detected in the intestine of resistant fish at 7-dpe (Cq of 27.0 ± 2.0 for resistant fish *vs*. a Cq of 24.8 ± 0.8 for susceptible fish). This further supports the hypothesis that resistant fish can recognize the parasite and elicit an immune response early in the infection that is capable of either directly killing the parasite or inhibiting its replication. It remains to be determined precisely when and where this occurs, but it may be occurring in the blood vessels during *C. shasta* migration from the gills to the intestine. When resistant and susceptible fish have been exposed in parallel to *C. shasta*, no difference in parasite burden has been detected in the gills, and both phenotypes had a suppressed IFN-γ response in this tissue (Bjork et al., 2014). Elimination of the parasite in the intestine doesn't seem to occur until much later in the infection (25+ dpe), and between 7- and 21-dpe, the amount of parasite DNA in the intestine seems to follow an exponential growth curve in both resistant and susceptible fish. Our observations suggest that initial resistance either occurs in the blood or when the parasite first reaches the intestine.

While it was not surprising to find a strong induction of IFN-γ, something that is commonly seen in *C. shasta* infected salmonids and other myxozoan infected fish (Baerwald, 2013; Bjork et al., 2014; Hurst et al., 2019; Jang et al., 2017), it was perhaps surprising that the immune response was so strongly focused on this one pathway. IFN-γ is the signature T_H1 cytokine and mediates the response to viruses and other intracellular pathogens. *C. shasta* is considered an extracellular pathogen and certainly occupies that role in the intestine. This raises the question as to how or why a pathway geared towards intracellular pathogens is invoked and why a cytosolic pathogen recognition receptor (NLRC5) would be upregulated. This may be explained by *C. shasta* having an intracellular phase early in the infection. The only evidence for this comes from a study by Bjork & Bartholomew 2010, which reported the invasion and migration of fluorescently stained *C. shasta* (Bjork and Bartholomew, 2010). The authors noted a potential intracellular phase in the endothelium of blood vessels starting at 3-dpe and suggested the use of electron microscopy to resolve the question of an intracellular developmental stage of the parasite. This would not be without precedent, as other myxozoans are known to have an early intracellular developmental stage or even having an intracellular final location. *M. cerebralis* initially replicates intracellularly in the epithelia of the epidermis of trout (El-Matbouli et al., 1995), and *Sphaerospora molnari* is intracellular in the gills of carp, prior to extracellular proliferation in the blood (Korytář et al., 2019), and *Kudoa* species develop within muscle fibres. In the latter case, it has been suggested that the parasite remains undetected by the host immune system due to their intracellular location, firmly enveloped by the remnants of the muscle fibre and the surrounding connective network of the endomysium (Kristmundsson and Freeman, 2014).

Whether an early intracellular phase is required for parasite development or represents a form of immune evasion remains to be determined. An intriguing possibility, at least for *C. shasta*, is that going intracellular causes the host to initiate a cytotoxic T_H1 response that is ineffective against the extracellular stages in the intestine. This is evidenced by the upregulation of antigen presentation machinery and MHC class I molecules, which are responsible for the presentation of cytosol-derived peptides to $CD8^+$ cytotoxic T lymphocytes (CTLs). Activated CTLs recognize and directly kill infected host cells, which is vital for controlling infections caused by intracellular pathogens (Janeway et al., 2001). A mistargeted immune response such as

this would explain why the rapid induction of IFN-γ in the intestine at 7-dpe failed to slow parasite proliferation, with significantly more parasite DNA being detected at 21-dpe.

If IFN-γ is playing a role in resistance to *C. shasta* in the intestine, the actual mechanism remains unclear. IFN-γ mediates parasite clearance by inducing the production of nitric oxide (NO) in classically activated macrophages (Bogdan et al., 2000; James, 1995). This antiparasitic effect has been demonstrated for numerous parasites, including the well-studied intestinal parasites *Entamoeba histolytica*, *Cryptosporidium parvum*, and *Giardia spp.* (James, 1995; Pavanelli et al., 2010; Zarebavani et al., 2017). In the present study, we did not observe upregulation of inducible nitric oxide synthase, which is responsible for NO production in macrophages, and instead observed upregulation of arginase at both 7- and 21-dpe. Arginase, a marker for alternatively activated macrophages, utilizes the same substrate as nitric oxide synthase (L-arginine) and redirects it to the formation of polyamines and proline, which are necessary for collagen synthesis and wound healing (Durante et al., 2007). In addition to depleting the necessary substrate for NO production, upregulation of arginase also inhibits the expression of inducible nitric oxide synthase. It is also curious that we observed upregulation of arginase at 7-dpe in the absence of any T_H2 cytokines, which typically drive its expression. The protozoan parasites *Trypanosoma brucei* and *T. cruzi* have both been shown to induce host expression of arginase in a T_H2-cytokine independent manner as a means of suppressing NO production (Aoki et al., 2004; De Muylder et al., 2013). Our observations may indicate either a protective host response geared towards controlling inflammation and maintaining tissue integrity, or a pathogenic strategy to escape IFN-γ driven NO production. Given how important the interplay between classically activated NO producing macrophages and alternatively activated arginase expressing macrophages are to the resolution of parasitic infections, an in-depth analysis of the macrophage populations during *C. shasta* infection would greatly benefit our understanding of the host immune response.

In addition to mounting an earlier immune response, resistant fish have a much different reaction to the parasite at the tissue level. At 7-dpe, evidence of tissue remodeling is present with the upregulation of junctional proteins, arginase, and the massive upregulation of caspase-14, which plays a terminal role in keratinocyte differentiation (Denecker et al., 2008). Keratins are cytoskeletal proteins classically used as epithelial cell markers during injury and disease in vertebrates (Ordóñez, 2013; Toivola et al., 2015) and which have also been described in non-

cornified mucosal epithelia of teleosts (Bunton, 1993). In mice, keratinocyte growth factor has been shown to ameliorate drug-induced intestinal mucosal disruption through induction of epithelial repair and tight junction protein expression, together with the inhibition of increased epithelial permeability (Song et al., 2020). The early changes in gene expression observed in this study, including the upregulation of caspase-14, point towards an active repair process of the intestinal barrier disrupted by the parasite. The gross pathology observed at 21-dpe, where the intestine took on a stiff, leathery appearance and was mechanically resistant to homogenization in liquid nitrogen might be related to the thickening of the lamina propria-submucosa due to the observed hyperplasia. The increased vascularization of the organ that we observed would contribute to the tissue repair process. By 21-dpe, no parasite stages were observed beyond the stratum compactum, which appeared to act as a parasite barrier. Thus, parasite proliferation is limited in resistant steelhead to the mucosal layers of the intestine, where cell regeneration occurs on a daily basis favoring lesion recovery. This is likely aided by the massive upregulation of IL-10 at 21-dpe, which would facilitate the resolution of inflammation and subsequent tissue repair. This is similar to what is observed in gilthead sea bream that become resistant to reinfection with *E. leei*, where IL-10 is associated with the resolution of infection (Picard-Sánchez et al., 2019). The tissue response of resistant steelhead in this study strongly contrasts with what is observed in susceptible fish, where all layers of the intestine become infected and it becomes a soft, spongy mass that loses its overall structure (Alama-Bermejo et al., 2019; Bartholomew et al., 1989; Bjork and Bartholomew, 2010). How the stratum compactum becomes a physical barrier and whether it contributes to the intestinal hardening, deserves further studies. It does appear that the ability of resistant fish to maintain their intestinal structure in the face of parasite replication is likely a critical factor in resisting *C. shasta* induced mortality and would explain why previous studies have observed organized, tissue-level responses to *C. shasta* in resistant fish, but not in susceptible fish (Bartholomew et al., 2004; Foott and Stone, 2004; Ibarra et al., 1991).

Differences in the intestinal epithelial integrity between hosts have also been observed in studies of the intestinal myxozoans *Enteromyxum scophthalmi* and *E. leei* (Ronza et al., 2020). Turbot is highly susceptible to *E. scophthalmi*, suffering serious intestinal lesions and barrier dysfunction, leading to high morbidity and mortality rates. Gilthead sea bream (*Sparus aurata* L.), on the other hand, experience low mortality rates and are able to better maintain their

intestinal epithelial integrity even when heavily infected by *E. leei* (Fleurance et al., 2008). Similar to *C. shasta*, *E. leei* is able to infect a wide range of fish species with varying degrees of host susceptibility (Sitjà-Bobadilla and Palenzuela, 2012). For sharpsnout seabream (*Diplodus puntazzo*), differences in humoral immune factors have been suggested to play a role in their high susceptibility to *E. leei* (Muñoz et al., 2007), however no transcriptomic comparison with gilthead sea bream has been conducted. It should also be noted that gilthead sea bream that survive primary exposure to *E. leei* acquire a protective immunity to reinfection, that is associated with increased IgM and IgT expression relative to naïve fish (Picard-Sánchez et al., 2019). Similar to gilthead sea bream, the resistant fish in this study were better able to maintain their intestinal integrity, and had higher expression of IgM and IgT compared to susceptible fish at this same point in the infection.

Given how different the intestinal response of resistant fish is at 7-dpe, compared to susceptible fish, it is surprising how similar the response is at 21-dpe. 7,820 genes were differentially expressed in both phenotypes and their expression levels were highly correlated. In addition to numerous metabolic and cell junction genes, this shared response includes several immune factors (IL-6, IL-8, IL-10, IFN-γ) that have been found to be upregulated in other studies of *C. shasta* infected salmonids (Bjork et al., 2014; Hurst et al., 2019; Taggart-Murphy, 2018). While we cannot rule out temporal differences in the expression of these genes being a critical factor in resistance, something that almost certainly occurs given the delayed parasite recognition of susceptible fish, their differential expression alone does not explain resistance vs. susceptibility to *C. shasta*. Examination of the genes that are only differentially expressed in resistant fish at 21-dpe revealed a much larger role for the adaptive immune response in these fish, particularly the B cell response. Resistant fish have significantly more heavy and light chain transcripts upregulated, as well as secreted IgM and IgT transcripts at this time. Again, this is likely influenced by the delayed parasite recognition of susceptible fish, but it suggests an earlier and stronger antibody response at play in resistant fish. It has been demonstrated that salmonids are capable of generating IgM and IgT that is specific to *C. shasta* (Zhang et al., 2010). However, the effectiveness of this antibody response in reducing mortality from *C. shasta* remains unclear. A study of susceptible rainbow trout exposed to *C. shasta* showed upregulation of IgM and IgT in these fish, but it failed to improve their condition and 100% mortality occurred (Taggart-Murphy, 2018). The authors noted that this may be due to the response

coming too late, after the intestine has been severely damaged. Our observations would support this, as resistant fish were better able to maintain their intestinal structure and mounted an earlier adaptive immune response, including the observed foci of lymphocyte-like cells.

Differences in the T cell response are also evident at 21-dpe, most notably among T_H17 cytokines which are an important aspect of the gut mucosal barrier that help prevent the dissemination of bacteria (Blaschitz and Raffatellu, 2010). In our previous study, we observed a strong downregulation of IL-17 family cytokines in susceptible fish at 21-dpe, whereas in the resistant fish in this study, no IL-17 cytokines were downregulated and IL-17A was upregulated. Whether this is directly related to the *C. shasta* is unclear and it may be more related to overall gut health and the invasion of opportunistic bacteria. More interesting is the possible transition to a T_H2 response that may be occurring late in the infection. This adaptive immune response is driven by IL4/13, which we observed massive upregulation of at 21-dpe, along with upregulation of the transcriptional regulators GATA3 and STAT5. The T_H2 response is associated with wound healing, the suppression of T_H1-driven inflammation, and the elimination of helminth parasites (Gause et al., 2013; Wynn, 2015). Although much smaller than helminth worms, *C. shasta* is also an extracellular intestinal parasite and the mechanisms used to clear helminths (B cell activation, eosinophil recruitment, mucus hypersecretion, increased cellular turnover) may help eliminate or otherwise create an unfavorable environment for *C. shasta*.

Comparison of our finding with other studies on myxozoan infections suggest that an effective T cell response is critical for mitigating disease. Similar to what we observe in *C. shasta* infected rainbow trout, when strains of rainbow trout that are resistant and susceptible to whirling disease are compared, the resistant strain has an earlier and more effective immune response, with upregulation of IFN-γ sooner in the infection and a stronger T cell response during the initial stages (Baerwald, 2013; Saleh et al., 2019). The failure of this early immune response in susceptible fish leads to a sustained inflammatory response that fails to control the infection and likely contributes to tissue damage. The pathology of proliferative kidney disease in rainbow trout is associated with an early imbalance of T_H cytokines and a dysregulated B cell response (Bailey et al., 2019). An in-depth analysis of T_H cytokines in gilthead sea bream during *E. leei* infection revealed a strong induction of T_H1 and T_H17 cytokines in the intestine (Piazzon et al., 2018). The ability of Atlantic salmon (*Salmo salar*) to resolve infection with *K. thyrsites*,

and develop resistance to reinfection, is linked to a cytotoxic T cell response (Braden et al., 2017).

CONCLUSIONS

In this study we employed RNA-seq to explore the complex dynamic between host and parasite and to understand how resistant steelhead are able to overcome an actively progressing *C. shasta* infection in their intestine. Our results confirm that early parasite recognition is critical for resistance and that initial invasion of the intestine by the parasite elicits a strong IFN-γ driven adaptive immune response in resistant fish. This is accompanied be remodeling of the host intestinal tissue which is likely vital for maintaining their overall intestinal structure in the face of extensive parasite replication and the tissue inflammation that results. This early adaptive immune response leads to a vigorous B cell response later in the infection, which is characterized by strong antibody production, particularly of IgT. Based on these results, we propose that a core immune response to *C. shasta* exists among resistant and susceptible fish and that temporal differences in the expression of key immune factors (IFN-γ, IL-6, GIMAP4, IgT), resulting from earlier parasite recognition by resistant fish, largely explains the different infection outcomes for these fish. In conjunction with this, resistant fish have a different response to the parasite at the tissue level with the stratum compactum playing an important role in limiting parasite spreading, which might be related to the induction of keratinization. Given that most RNA-seq studies have been conducted on fish that are susceptible to myxozoans, we believe the present study offers a valuable framework for putting those, and future studies, in perspective.

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CHAPTER 4: GENOME-WIDE SCAN IDENTIFIES A REGION OF OMY9 WITH A MAJOR EFFECT ON RESISTANCE TO THE PARASITE *CERATONOVA SHASTA* IN RAINBOW TROUT

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ABSTRACT

Parasites and the diseases they cause are a major threat to fish welfare, threating the stability of many aquaculture systems, and the conservation of wild fish stocks. Ceratomyxosis, caused by the intestinal parasite *Ceratonova shasta*, is an important disease of Pacific salmonids that leads to severe intestinal inflammation and potentially death of the fish host. There are no viable treatments for ceratomyxosis, and the disease has been linked to population level declines of certain fish stocks. Management of disease relies on selective stocking of fish that are genetically fixed for resistance to ceratomyxosis. To identify the genetic basis of this resistance, a genome-wide scan was conducted on 6,809 SNPs generated from Double-Digest Restriction Associated DNA (ddRAD) sequencing of 84 individuals from a backcross family of rainbow trout. A region on chromosome Omy9 was identified as being significantly associated with surviving infection, and another region on Omy11 was identified that had a lesser contribution to survival. The identification of single genomic region with a large effect on surviving ceratomyxosis has important implications for the potential of selective breeding and moves us closer to a mechanistic understanding of resistance to this important pathogen.

INTRODUCTION

Parasitic diseases represent a significant threat to the welfare of fish worldwide, negatively impacting fish in both the wild and aquaculture setting. Globally, aquaculture is one of the fastest growing food production sectors (Ahmed et al., 2019). However, the sustainability of many aquaculture production systems is threated by parasitic diseases that lead to pre-harvest mortality, reduced yield, or reduced marketability (Costello, 2009; Henning et al., 2013; Shinn et al., 2015a). It has been estimated that the global burden that parasites place on finfish production results in losses up to $9.6 billion U.S. dollars (Shinn et al., 2015b). Management of parasites in aquaculture often relies on chemical therapies, which may have negative impacts on the environment and human health (Cole et al., 2009; Burridge et al., 2010; Rico et al., 2012; Langford et al., 2014). Beyond aquaculture, the conservation of wild fish stocks is also negatively affected by parasitic disease (Bruneaux et al., 2017; Krkosek et al., 2006; Krkošek et al., 2007, 2013). Selective stocking of genetically resistant fish strains for conservation biology, or the use of selective breeding programs for disease resistance in aquaculture, offers an attractive alternative for mitigating parasite impacts. Understanding the genetic basis of disease resistance is vital for this, as disease resistance may be negatively correlated with other important

traits, and it facilitates the use of marker assisted selection to identify fish with multiple desirable traits for breeding (Yue, 2014; Yáñez et al., 2016; Eze, 2019).

Rainbow trout (*Oncorhynchus mykiss*), and other Pacific salmonids, are amongst the most widely cultivated fish species in the world (FAO Fisheries). Throughout their native range, Pacific salmon and trout are of economic, ecological and cultural importance (Criddle and Shimizu, 2014; Lichatowich and Lichatowich, 2001). However, many fish stocks, both wild and hatchery-reared, are negatively impacted by parasites (Moran et al., 1999; Jacobson et al., 2008; True et al., 2016). One of the most virulent is *Ceratonova shasta*, an intestinal myxozoan parasite of salmon and trout that causes ceratomyxosis, a disease characterized by severe inflammation, hemorrhaging, and necrosis of the intestine (Bartholomew et al., 1989; Bjork and Bartholomew, 2010; Wales and Wolf, 1955). *C. shasta* is enzootic in many watersheds throughout the Pacific Northwest of the Unites States and Canada (Bartholomew, 1998; Hoffmaster et al., 1988), where it infects a broad range of salmonid species (Stinson et al., 2018). *C. shasta* has been linked to population level declines of certain wild fish stocks, and epizootics are known to occur in the hatchery setting (Sanders et al., 1972; Bartholomew et al., 2004; Fujiwara et al., 2011; Hallett et al., 2012).

While both biotic and abiotic factors influence the outcome of infection, the largest determinant is the innate resistance of the host (Bartholomew, 1998; Hallett et al., 2012). Allopatric fish populations are highly susceptible to ceratomyxosis, with a single spore capable of causing a lethal infection (Bjork and Bartholomew, 2009; Buchanan et al., 1983; Ratliff, 1983; Zinn et al., 1977). This strongly contrasts with sympatric fish populations, which have acquired a high degree of resistance to the disease (Ibarra et al., 1992; Bartholomew, 1998; Ray et al., 2010). Numerous inheritance studies conducted on both sympatric and allopatric fish populations have demonstrated that resistance is a highly heritable trait that shows little variation within a given population (Buchanan et al., 1983; Hemmingsen et al., 1986; Ibarra et al., 1992, 1994; Bartholomew et al., 2001; Nichols et al., 2003). When allopatric and sympatric fish are crossed, the offspring have a *C. shasta* induced mortality threshold intermediate that of the parents, indicating that the alleles conferring resistance are dominant and being homozygous at these loci confers an advantage (Ibarra et al., 1994).

Currently, no therapeutic or prophylactic treatment exists for ceratomyxosis, and management of the disease relies on selective stocking of resistant fish strains. However, even

resistant fish can succumb to the infection, and determining the resistance threshold of a fish stock is experimentally difficult and has not been done for most stocks (Ray et al., 2010). Understanding the genetic and molecular basis of resistance would provide the groundwork for the development of therapeutics, and the identification of genetic markers for resistance would provide a non-lethal means of assessing a stock's resistance. A previous genetic mapping study identified multiple genetic loci associated with resistance to *C. shasta* in rainbow trout (Nichols et al., 2003). However, this study was hampered by a small number of individuals in the analysis and lacked both a reference genome and sequencing data. The authors utilized amplified fragment length polymorphic markers to identify several linkage groups associated with resistance in doubled haploid progeny that were produced by androgenesis from an F1 hybrid cross of resistant and susceptible fish. This work confirmed that the trait was polygenetic but was unable to identify specific loci conferring resistance.

Our research group expanded upon this work by preforming a comparative transcriptomic analysis of resistant and susceptible steelhead exposed to *C. shasta* (chapter 2). This identified parasite recognition as a critical factor in resistance, as susceptible fish were unable to recognize the initial invasion of *C. shasta* within the intestine. The analysis also identified two potential candidate resistance genes (NLRC5, GIMAP4), that were highly upregulated in resistance fish and have known immune functions. A subsequent study examined the response of resistance steelhead with a heavy parasite burden using both transcriptomics and histopathology (chapter 3). This revealed that resistant fish have a much different response to *C. shasta* at the tissue level, with the stratum compactum of the intestine acting as a barrier, preventing migration into deeper layers, and limiting the spread of the parasite to the mucosal surfaces of the intestine. This coincided with the massive upregulation of caspase-14 in the intestine, which plays a terminal role in keratinocyte differentiation. While these genes appear to play a role in resistance to *C. shasta*, whether or not they are the actual cause of resistance cannot be determined without identifying the genomic regions that confer resistance.

In this study, we sought to identify and map the genomic regions associated with resistance to *C. shasta* induced mortality in rainbow trout. In order to do this, we created a backcross generation of fish in which the resistance alleles are segregating. We employed RAD-seq to genotype resistant and susceptible offspring and identify single-nucleotide polymorphisms (SNPs) that can be tested for association with resistance. We then compared the genes within

these regions to the genes we had previously found to be upregulated in response to *C. shasta* infection.

MATERIALS AND METHODS

Creation of backcross

An F1 hybrid generation was created by crossing resistant redband rainbow trout with susceptible Roaring River rainbow trout. Two wild redband males, obtained from the Klamath River with the assistance of the Oregon Department of Fish Wildlife, were crossed with two female rainbow trout from the Roaring River Hatchery. The offspring were reared under specific-pathogen free (SPF) well water at the Oregon State University John L. Fryer Aquatic Animal Health Laboratory (AAHL). After two years, three sexually mature F1 males were chosen at random and crossed with a female Roaring River rainbow trout to create three half-sib backcross families. Tissue samples were collected from each of the parents after the cross and stored at -80° C for later genotyping. The eggs from each family were incubated on separate racks of a vertical flow incubator until hatching. Hatching rates were similar across the three families (>90%) and they were combined into one tank after hatching. At three months post hatch, approximately 700 fish (~10 g) were transferred to a separate 678-liter tank and allowed to acclimate for three weeks prior to initiation of the parasite challenge.

Parasite exposure and phenotyping

The source of *C. shasta* actinospores for this challenge came from a laboratory culture of the invertebrate host *Manayunkia occidentalis* (Atkinson et al., 2020; Bartholomew et al., 1997) infected with *C. shasta* genotype IIC, which are maintained in indoor mesocosms at the AAHL. 24-hours prior to the exposure, the incoming water to the mesocosm was turned off to allow the actinospores to accumulate in the mesocosm water. The challenge was initiated by turning off the incoming water to the exposure tank and adding 1-liter of mesocosm water. The fish were held on static water with aeration for 22-hours, at which point the incoming water was turned back on. To determine the exposure dose, 1-liter water samples were collected from the exposure tank 3 minutes after adding the mesocosm water and just prior to turning the water back on. The water samples were filtered and prepared for qPCR to quantity the number of *C. shasta* spores per liter of water, following a previously described method (Hallett and Bartholomew, 2006; Hurst and Bartholomew, 2012).

The fish were monitored daily for mortality and those exhibiting clinical signs of ceratomyxosis (lethargy, lack of appetite, abdominal bloating) were euthanized with an overdose of MS-222 (tricaine methanosulfonate, Argent Laboratories, Redmond, WA, USA). Intestinal scrapings were collected from each deceased fish with a sterile loop and examined under a microscope for the presence of *C. shasta* myxospores or pre-sporogonic stages. If present, the fish was recorded as susceptible and a muscle tissue sample was collected and immediately frozen. Fish that perished but had no visible *C. shasta* spores in their intestine were marked inconclusive and excluded from the analysis. Monitoring of the fish continued until no mortalities were recorded for a 45-day period, at which time all remaining fish were euthanized with an overdose of MS-222. The fish that survived until this point were considered resistant if no clinical disease signs were present and no *C. shasta* spores were detected. Muscle tissue samples were collected from 96 resistant fish, chosen at random, and immediately frozen.

DNA extraction and sequencing

DNA was extracted from 88 samples for genotyping: the 4 parents, and 42 each of the resistant and susceptible offspring, chosen at random. Genomic DNA was extracted using the DNeasy Blood & Tissue Kit (Qiagen, catalog number 69506), according to the manufactures protocol. The purity of each DNA sample was assessed with a NanoDrop One UV-Vis Spectrophotometer and the integrity checked via agarose gel electrophoresis. The samples were submitted to the Center for Genome Research and Biocomputing at Oregon State University for ddRAD (Peterson et al., 2012) genotyping with *SbfI* and *MspI* and pooled into one library for sequencing on one 100-bp single end lane on an Illumina HiSeq 3000.

Quality filtering and SNP calling

The raw sequencing reads were trimmed of adapter sequences using BBDuk (January 25, 2018 release, https://jgi.doe.gov/data-and-tools/bbtools/) and reads less than 10-bp after trimming were discarded. The reads were assessed for quality before and after trimming using FastQC (v 0.11.8). The trimmed reads were processed using the *STACKS* pipeline (v 2.52) (Paris et al., 2017; Rochette and Catchen, 2017) as follows. The *process_radtags* module was used to demultiplex the reads and assign them to individual samples based on the presence of unique in-line barcodes (Peterson et al., 2012). The demultiplexed reads were then mapped to the rainbow trout reference genome (RefSeq: GCF_002163495.1) using BWA (v 0.7.17) (Li and Durbin, 2009). The *gstacks* module was used to assemble the reads into loci and call SNPs. For the

populations module, the samples were grouped into resistant and susceptible populations and the loci were filtered and retained only if they were: present in both populations (-p 2), present in at least 80% of individuals (-r 0.8), had a minor allele frequency greater than 5% (--min-maf 0.05), and had a maximum heterozygosity less than 0.65 across both populations (--max-obs-het 0.65). Population genetic statistics were also calculated (--fstats -k --sigma 100000) and the data exported in VCF and Plink format for downstream analysis. Prior to association testing, Plink (v 1.90b5.2) (Purcell et al., 2007) was used to exclude individuals if they had more than 10% missing genotype data (--mind 0.1) and exclude SNPs if they were missing in more than 2% of samples (--geno 0.02) or failed the Hardy-Weinberg test (--hwe 0.000001).

Association testing and identification of candidate genes

Resistance to *C. shasta* was treated as a binary variable (1 = susceptible, 2 = resistant) and each RAD locus retained after filtering was tested for a correlation with resistance using logistic regression (--logistic) in Plink and Bonferroni and Benjamini-Hochberg adjusted p-values were calculated for each significance test using the --adjust option. Association mapping results were imported into R (v. 4.0.0) and visualized using the R package qqman (D. Turner, 2018). The extent of linkage disequilibrium (LD) among statistically significant SNPs was assessed with Plink and expressed as the correlation coefficient R^2. Haplotype blocks were identified as SNPs having pairwise LD of $R^2 = 1$, indicating mutually perfect LD. Potential candidate genes were identified by comparing the list of genes found within 1 Mb of the identified haplotype blocks to a set of genes previously found to upregulated in response to *C. shasta* infection in resistant steelhead (Chapters 2 & 3).

RESULTS

Parasite exposure and phenotyping

Waters samples taken at the beginning and end of the exposure indicate there was initially 50 spores per liter of water in the exposure tank, which dropped to 2 spores per liter by the end of the exposure. Based upon the volume of water in the tank (678 L), this equates to approximately 33,900 total spores and a dose of 53 spores per fish. Mortality from *C. shasta* began to occur 46-days post exposure (dpe), and continued until 130-dpe, when the last mortality was recorded. The exposed fish either developed strong clinical signs of ceratomyxosis; anorexia, severe abdominal bloating, lethargy, and a bloody vent, or showed no clinical signs of disease. A total of 159 fish succumbed to the infection, representing 25% of the fish exposed (Fig. 4.1A). This indicates that two loci conferring resistance to *C. shasta* are segregating within this population, and fish that possess at least one have increased resistance to *C. shasta* induced mortality. This is consistent with the F1 hybrid parent being heterozygous at both loci, and the susceptible parent being homozygous at both loci (Fig. 4.1B), resulting in the observed ratio of resistant to susceptible offspring (Fig. 4.1C).

A)

Backcross offspring phenotyping	
Resistant	458 (72%)
Susceptible	159 (25%)
Inconclusive	21 (3%)
Total	638

B)

Putative parental genotypes	
F1 Hybrid	AaBb
Susceptible	aabb

C)

	AB	Ab	aB	ab
ab	AaBb	Aabb	aaBb	aabb
%	75%			25%

Figure 4.1. Inheritance pattern of ceratomyxosis resistance alleles. A) shows the results from phenotyping the backcross offspring. B) Putative parental genotypes that would explain the observed inheritance of the resistance trait. C) Punnett square showing the inheritance of two alleles from the putative parental genotypes.

SNP discovery and association with resistance

Double-digest RAD seq was employed to genotype resistant and susceptible fish for association testing. After processing the sequencing reads through the Stacks pipeline and Plink, a total of 6,809 SNPs were retained. Association testing identified 54 SNPs significantly associated with *C. shasta* resistance at the Bonferroni adjusted p-value threshold (7.34×10^{-6}), all of which were located on chromosome Omy9 (Fig. 4.2). Another 93 SNPs were significant at the less strict Benjamini-Hochberg adjusted threshold (9.50×10^{-4}) and were located on chromosomes Omy9 and Omy11. A subset of the of the statistically significant SNPs are listed in Table 4.1.

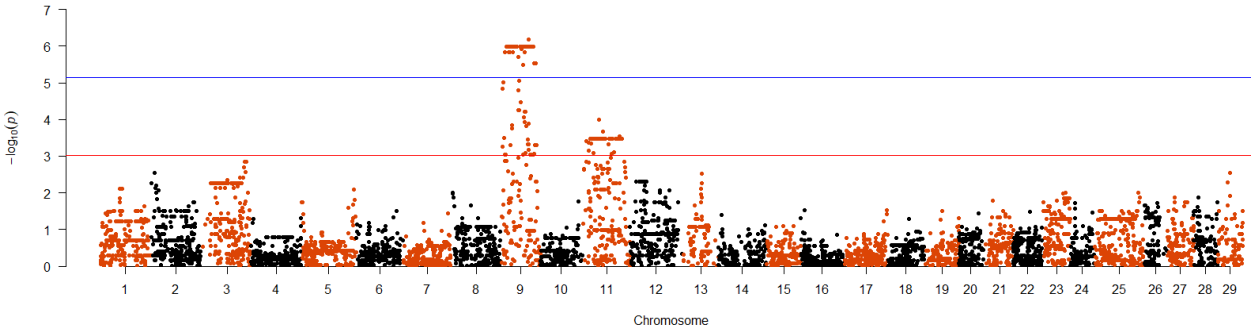

Figure 4.2. Manhattan plot showing the genome-wide significance of each SNPs association with resistance to *Ceratonova shasta*. The x-axis shows the genomic location of each SNP and the y-axis is the -log10 transformed p-value. The blue line indicates the Bonferroni adjust significant threshold and the red line indicates significance as at the Benjamini-Hochberg threshold

Table 4.1. Single-nucleotide polymorphisms (SNPs) significantly associated with resistance to *Ceratonova shasta* induced mortality. † denotes SNPs that are part of large haplotype blocks.

SNP ID	Chr	Position	Reference allele	Alternative allele	Heterozygosity - susceptible	Heterozygosity - resistant	$-\log_{10}P$	Odds-ratio
164313_13	9	48373112	G	A	0.093	0.698	6.18	22.75
154418_45†	9	10604865	T	G	0.093	0.667	5.99	21.00
166012_46†	9	55932913	T	G	0.093	0.667	5.99	21.00
161258_59	9	36546111	A	C	0.071	0.667	5.91	28.00
154054_33	9	8611317	T	G	0.093	0.659	5.84	20.25
155523_49	9	15434667	T	C	0.093	0.659	5.84	20.25
155523_69	9	15434687	G	T	0.093	0.659	5.84	20.25
155681_23	9	16087688	T	C	0.093	0.659	5.84	20.25
157025_7	9	21045148	A	T	0.093	0.659	5.84	20.25
157025_53	9	21045194	A	G	0.093	0.659	5.84	20.25

157251_53	9	21965796	G	A	0.093	0.636	5.84	20.25
162572_39	9	41564504	G	A	0.093	0.659	5.84	20.25
162625_11	9	41735159	A	T	0.093	0.659	5.84	20.25
159719_38	9	31354268	G	A	0.093	0.600	5.70	19.07
166375_66	9	57585805	A	G	0.140	0.659	5.53	13.28
166478_69	9	58158214	A	G	0.140	0.667	5.53	13.28
166478_43	9	58158240	T	C	0.140	0.667	5.53	13.28
166686_20	9	59135822	A	C	0.140	0.667	5.53	13.28
166809_83	9	59757455	A	T	0.140	0.667	5.53	13.28
166835_89	9	59871292	A	G	0.140	0.667	5.53	13.28
161849_30	9	38724257	G	A	0.000	0.667	5.48	4.53
186187_51†	11	13634383	C	T	0.256	0.659	3.48	5.61
186372_80†	11	14325133	G	C	0.256	0.644	3.48	5.61
199726_43†	11	67156601	G	A	0.256	0.644	3.48	5.61

Identification of resistance haplotypes

Linkage disequilibrium analysis identified a set of 35 SNPs on chromosome Omy9 showing perfect pairwise LD ($R^2 = 1$) (Fig. 4.3). Each SNP was associated with resistance to *C. shasta* at the same significance level ($p = 1.03 \times 10^{-6}$) and had the same level of heterozygosity among resistance (0.667) and susceptible (0.093) fish. Logistic regression indicated that heterozygous fish were 21 times more likely to survive *C. shasta* infection than homozygous fish (odds-ratio = 21, 95% CI: 6.19 to 71.22). A similar pattern was observed among a set of 33 SNPs on chromosome Omy11, with each SNP showing perfect pairwise LD ($R^2 = 1$) and having the same significance level ($p = 3.29 \times 10^{-4}$) (Fig. 4.4). Susceptible fish had an observed heterozygosity of 0.256 at each of these loci, whereas heterozygosity varied from 0.644 to 0.659 in resistant fish. Heterozygous fish were over five times more likely to survive *C. shasta* infection than homozygous fish (odds-ratio = 5.61, 95% CI: 2.19 to 14.38). Additionally, at each of the 68 polymorphic sites described above, the susceptible parent was homozygous at each site and all three resistant parents were heterozygous at each site.

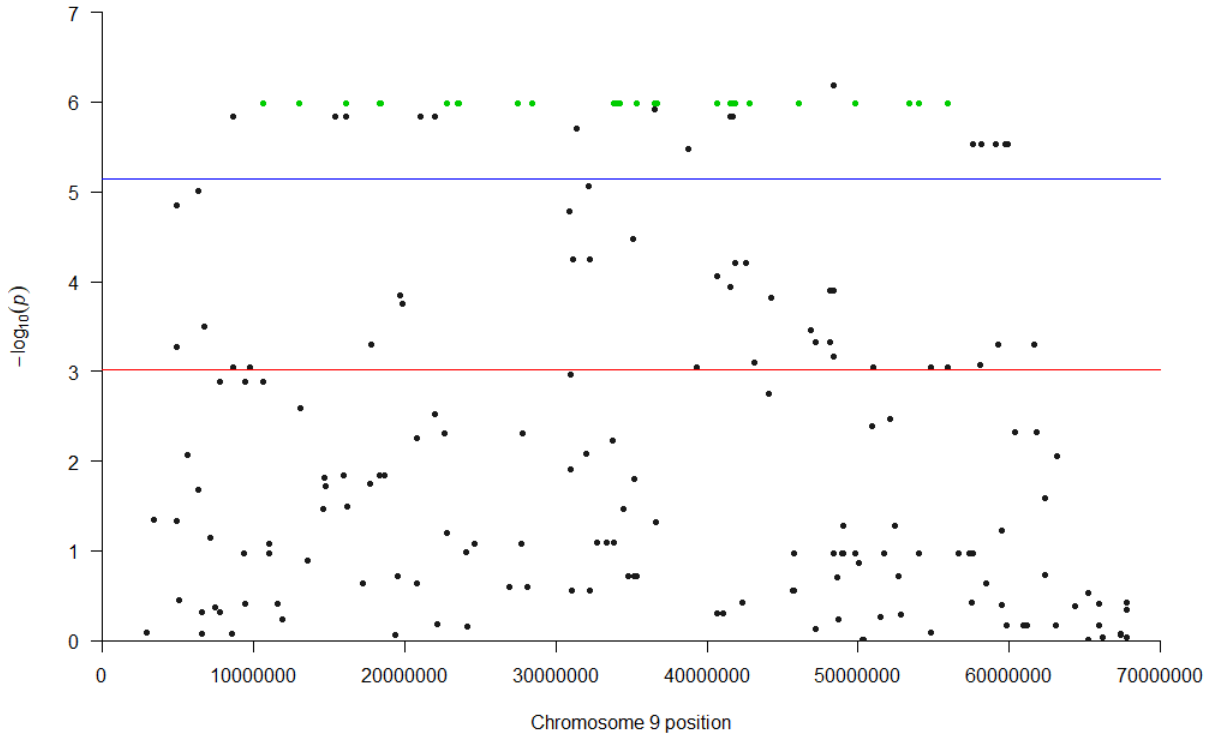

Figure 4.3 Manhattan plot showing each SNP located on chromosome Omy9. X-axis indicates the genomic position of the SNP and the y-axis indicates the statistical significance of its association with resistance to *C. shasta* induced mortality. SNPs highlighted in green exhibit perfect pairwise LD (R2 = 1). The blue line indicates the Bonferroni adjust significant threshold and the red line indicates significance as at the Benjamini-Hochberg threshold.

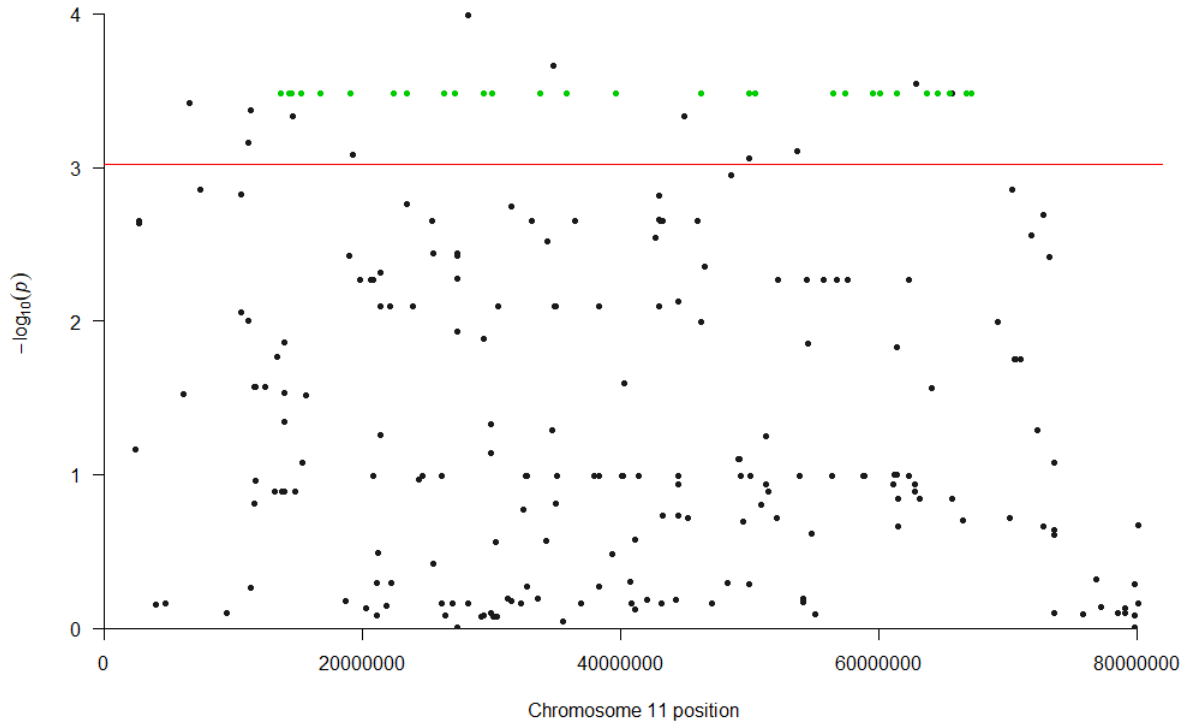

Figure 4.4 Manhattan plot showing each SNP located on chromosome Omy11. X-axis indicates the genomic position of the SNP and the y-axis indicates the statistical significance of its association with resistance to *C. shasta* induced mortality. SNPs highlighted in green exhibit perfect pairwise LD (R2 = 1). The blue line indicates the Bonferroni adjust significant threshold and the red line indicates significance as at the Benjamini-Hochberg threshold.

Identification of candidate genes

Due to the extent of long-range LD present within this population, we considered all genes on Omy9 and Omy11 as potential candidate genes. However, from this list of genes, two subsets were created for:

1) Genes that were previously found to be upregulated in resistant steelhead after infection with *C. shasta* (chapters 2 & 3), which identified 199 genes on Omy9 and 186 on Omy11 (supplementary).

2) Genes that were upregulated and located within 1 Mb of the identified haplotype blocks (between base pairs 9604865-56932913 on Omy9, and 12634383-68156601 on Omy11), which identified 122 genes on Omy9 and 132 on Omy11 (supplementary).

From these lists, a subset of genes that have known immune functions or that could be related to the distinct tissue response observed in resistant steelhead (chapter 3) were identified and are presented in table 4.2.

Table 4.2. Candidate resistance genes. Rows in bold indicate genes that are located within the identified haplotype blocks.

Chromosome	Start	Stop	Entrez Gene Id	Length	Protein Name	Upregulated in response to *C. shasta*
Omy9	6948434	6949252	LOC110531510	178	C-type lectin-like	No
Omy9	7009082	7017534	LOC110531495	1111	protein KHNYN-like/Fucolectin-	Yes
Omy9	7025799	7026868	LOC110531512	129	L-selectin-like	No
Omy9	7029298	7030078	LOC110532921	161	fucolectin-1-like, partial	No
Omy9	7044430	7049141	LOC110531506	334	C-type mannose receptor 2-like	No
Omy9	7307071	7342975	LOC110532924	165	fucolectin-like	No
Omy9	7988578	7990195	LOC100301641	252	mannan-binding lectin H1 precursor	No
Omy9	8020603	8027407	LOC110532929	159	fucolectin-4-like	No
Omy9	8035750	8039207	LOC110531541	355	integumentary mucin C.1-like	No
Omy9	9096004	9105420	LOC110533001	436	GTPase IMAP family member 7-like	Yes
Omy9	**12500004**	**12505122**	**nitr2**	**363**	**novel immune-type receptor 2**	**No**
Omy9	**12563446**	**12565117**	**nitr3**	**356**	**novel immune-type receptor 3**	**No**
Omy9	**13197151**	**13203393**	**LOC110531694**	**96**	**high affinity immunoglobulin epsilon receptor subunit gamma-like**	**Yes**
Omy9	**14746002**	**14750763**	**LOC110531734**	**202**	**leucine rich adaptor protein 1-like**	**Yes**
Omy9	**28731792**	**28739014**	**LOC110531965**	**263**	**periphilin-1**	**Yes**
Omy9	**29933150**	**29977230**	**LOC110531988**	**1233**	**fibulin-2-like**	**Yes**
Omy9	**50780155**	**50782387**	**LOC110532464**	**288**	**tumor necrosis factor receptor superfamily member 9-like**	**Yes**
Omy9	**52866229**	**52948604**	**LOC110532566**	**1054**	**integrin alpha-5-like**	**Yes**
Omy11	11938317	11944218	LOC110535315	412	interferon regulatory factor 8-	Yes
Omy11	**18142303**	**18153722**	**cd226**	**334**	**CD226 antigen**	**Yes**
Omy11	**32090444**	**32096760**	**LOC110535615**	**271**	**GTPase IMAP family member 4-like**	**No**
Omy11	**46484674**	**46504072**	**LOC100136247**	**532**	**CD36 antigen**	**Yes**
Omy11	73747762	73768596	LOC110535123	960	endoplasmic reticulum aminopeptidase 1-like	Yes

DISCUSSION

In this study, we created a backcross generation of fish in which the alleles conferring resistance to *C. shasta*-induced mortality were segregating. By genotyping resistant and susceptible offspring, we identified two genomic regions strongly associated with the resistant phenotype. A large haplotype block on chromosome Omy9 was associated with a 21-fold increase in the odds of surviving *C. shasta* infection and another large haplotype block on chromosome Omy11 was associated with a 5-fold increase in survival. Genes located within these genomic regions were compared to a set of genes previously found to be upregulated in resistant steelhead upon infection with *C. shasta*, identifying a set of 254 potential candidate resistance genes.

Phenotyping the backcross offspring indicated that two independent loci were present within the population that confer increased resistance to *C. shasta* induced mortality. This is consistent with earlier research that indicated that resistance was a polygenic trait (Ibarra et al., 1994; Nichols et al., 2003). Based upon this Mendelian inheritance, we would expect that two-thirds (67%) of the resistant offspring would be heterozygous at each loci (Figure 1C) and that susceptible fish would be homozygous at both loci. In agreement with this, the two haplotype blocks we identified as being associated with resistance had an observed heterozygosity at or very near 67% in resistant fish (Table 4.1). Interestingly, susceptible fish had an observed heterozygosity of 9% for the haplotype block on Omy9 and a heterozygosity of 25% for the haplotype block on Omy11. This suggests that the resistance allele on Omy11 has a weaker effect on resistance, which aligns with the lower odds-ratio reported for SNPs in this region. Although we cannot determine the number of genes involved, a single region on Omy9 greatly increasing the odds of surviving *C. shasta* infection has important implications for the potential of selective breeding for resistance or the development of genotyping assays for the assessment of resistance.

The genomic regions we identified in this study align with a previous genetic mapping experiment which identified several linkage groups associated with resistance to *C. shasta* in rainbow trout that were later shown to be on chromosomes Omy2, Omy9, Omy16, and Omy20, as well as a notable marker on Omy11 (Nichols et al., 2003; Phillips et al., 2006). The additional genomic regions found in that study are partially explained by the different statistical tests they used, including testing for association with both binary survival and days to death. It is

noteworthy that the source of resistance genes in the aforementioned study came from Clearwater steelhead from the Dworshak National Fish Hatchery (Ashakha, Idaho). These fish are phylogenetically distinct from the fish used in the current study and are from a different watershed. Although more fish populations will need to be tested, the identification of resistance loci on chromosomes 9 and 11 in distinct fish populations suggest a conserved mechanism may be present among Pacific trout.

In a separate genetic mapping study conducted on rainbow trout, a single QTL on Omy9 was found to explain most the variance in resistance to whirling disease, caused by another myxozoan parasite, *Myxobolus cerebralis* (Baerwald et al., 2011). The resistant fish strain used in that study was the Hofer strain, which was introduced into Germany in the late 1800's and has since developed resistance to whirling disease. An intriguing possibility is that genes conferring resistance to whirling disease evolved from ancestral genes that conferred resistance to another myxozoan parasite, *C. shasta*. The exact origins of the Hofer strain are unclear, but most rainbow trout introduced to Europe appear to originate from hatcheries in Northern California, in particular the Baird Station on the McCloud River (Stanković et al., 2015, 2016). The McCloud River is a tributary to the Pit River, which feeds into the Upper Sacramento River, a *C. shasta* endemic watershed (Hendrickson et al., 1989; Ibarra et al., 1991). While we cannot say with certainty, but it is likely that the Hofer strain originated from a stock of rainbow trout with historical exposure to *C. shasta*. If the genes that confer resistance to whirling disease evolved from *C. shasta* resistance genes, then identifying the causal genes for resistance to one parasite would also mean identifying the resistance genes for the other parasite. It has been demonstrated that resistance to ceratomyxosis does not confer resistance to whirling disease (Hedrick et al., 2001), however, it is not known if the opposite is true.

A primary goal of this study, in addition to identifying genomic regions associated with resistance, was to identify a set of candidate resistance genes. This was somewhat hampered by the large genomic regions identified. Chromosomes Omy9 and Omy11 contain 1,450 and 1,346 genes, respectively. The causal genes could potentially be anywhere on these chromosomes, given the extent of long-range LD present in this population, but narrowing the region down to ± 1 Mb of the identified haplotype blocks provides us with a smaller region of higher confidence. We attempted to narrow the list down further by identifying genes within the region that were also upregulated in response to *C. shasta* infection in our prior transcriptomic studies (chapters 2

& 3). However, this cannot rule any genes out as 1) transcription of the casual genes may occur in an organ other than the gills or intestine or at a different time in the infection, 2) the genes may be constitutively expressed at a constant level, or 3) the fish used in the transcriptomic study may be resistant through a different mechanism. There is also the potential that something other than a gene is conferring resistance, such as a cis-regulatory element. With that being said, identifying a gene that is both upregulated in response to *C. shasta* infection and located within the identified genomic regions would offer robust support for its role in resistance to ceratomyxosis.

One such gene is *FCER1G*, which encodes high affinity immunoglobulin epsilon receptor subunit gamma, an adapter protein that transduces activation signals from various immunoreceptors, including B- and T-cell antigen receptors and C-type lectin receptors, leading to activation of the NF-kB inflammatory pathway (Qian et al., 1993; Lawrence, 2009; van der Poel et al., 2011; Bournazos et al., 2015; Guo et al., 2018). This gene was upregulated in resistant steelhead at both 7- and 21-days post exposure (dpe) to *C. shasta* (chapter 3). While it is located 6 Mb from the identified haplotype block on Omy9, a gene encoding GTPase IMAP family member 7 was also highly upregulated in resistant steelhead at both timepoints in our previous study (chapter 3). This is noteworthy, as we had identified two copies of GTPase IMAP family member 4 that were the most highly upregulated immune genes in the gills of resistant steelhead at 1-dpe and later in the intestine of susceptible steelhead at 14-dpe (chapter 2), suggesting an important role in resistance to *C. shasta*. GTPase immunity-associated proteins (GIMAPs) are a conserved family of GTPases that play a role in T-lymphocyte development and activation, and were recently shown to mediate resistant to the parasite *Toxoplasma gondii* in the Lewis rat strain, which is extremely resistant to the parasite (Filén and Lahesmaa, 2010; Kim et al., 2018).

While they were not found to be upregulated in response to *C. shasta* infection in our previous studies, the *NITR* gene cluster located within the identified haplotype block on Omy9, are perhaps the most promising candidate resistance genes. These genes encode novel immune-type receptors (NITRs), which are a family of transmembrane proteins that contain an immunoglobulin domains and immunoreceptor tyrosine-based inhibition motifs (Yoder et al., 2002). This gene family appears to have undergone a large expansion in teleost fish, with

numerous copies being identified in the genomes of Southern pufferfish (*Spheroides nephelus*), zebrafish (*Danio rerio*), European sea bass (*Dicentrarchus labrax*), rainbow trout, and several other teleosts (Ferraresso et al., 2009). This is consistent with other innate immune receptors, which have similarly undergone large expansions in teleost fish (Tørresen et al., 2018; Wcisel and Yoder, 2016), likely due to the greater reliance that poikilotherms have on the innate immune system (Jones, 2001). What is most intriguing about this gene cluster is that it is present on the Omy9 linkage group that had previously been associated with resistance to *C. shasta* in rainbow trout (Nichols et al., 2003). An innate immune receptor that has evolved to specifically recognize *C. shasta* would explain the differences in parasite recognition that are observed between resistant and susceptible steelhead (chapter 2), and would make sense given the long co-evolutionary history of fish and myxozoans (Holzer et al., 2018). It is possible that our previous RNA-seq analysis missed the window in which upregulation of these genes occurs. A study conducted on Zebrafish (*Danio rerio*) inoculated with *Listonella anguillarum* found rapid induction of *NITR9* gene expression at 2-hours post infection with expression levels decreasing afterwards (Rojo et al., 2007).

In addition to identifying potential candidate genes, it is also important to highlight the genes that were not found within the identified genomic regions. Most notably, no Nod-like receptors (*NLRC*) genes are present in these regions. In our previous comparative transcriptomics study, *NLRC5* was upregulated in both the gills and intestine of resistant steelhead after exposure to *C. shasta* (chapter 2). An innate immune receptor whose increased expression tracked with the infection offered a promising candidate gene. While *NLRC5* seems to play a role in the immune response to *C. shasta* in resistant fish, it does not appear to be the proximate cause of resistance, at least in this cross. This is an important distinction to make, as the response to a pathogen is a complex biological process involving hundreds of genes, most of which are going to be identical between resistant and susceptible fish, while only a limited number of genes are going to cause resistance and differ between the two phenotypes. A similar observation was made for resistance to whirling disease, where a microarray analysis identified *metallothionein B* (*Met B*) as candidate resistance gene, being highly upregulated in the resistant Hofer strain (Baerwald et al., 2008), but it was not present within the QTL region associated with resistance to whirling disease (Baerwald et al., 2011).

CONCLUSIONS

In the work presented here, we have identified a region on Omy9 that is significantly associated with resistance to *C. shasta*-induced mortality, and another region on Omy11 with a lesser contribution to survival. Two other genetic mapping studies have also identified regions on Omy9 as being associated with resistance to a myxozoan disease, suggesting that a conserved mechanism is at play or that Omy9 is enriched for myxozoan disease resistance genes. From this region, we were able to identify a set of candidate resistance genes, although finer mapping will need to be done to further narrow down the region and the list of candidates. Future studies could perform deep sequencing of Omy9 from multiple resistant and susceptible fish populations to identify genomic regions that are highly diverged between the two phenotypes. This would also identify genetic markers that could be used to assess the resistance level of a fish stock or utilized in marker-assisted selection. Looking beyond *O. mykiss*, it remains to be determined if all Pacific salmonids are resistant through the same mechanism, or if multiple mechanisms have evolved independently. The work presented here lays the foundation for answering such questions.

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CHAPTER 5: SUMMARY

The goal of this dissertation was to understand the immunological and genetic basis of resistance to the parasite *Ceratonova shasta* in *Oncorhynchus mykiss* (rainbow trout and steelhead), which was pursued through a series of comparative transcriptomic and genomic studies.

Beginning at the portal of entry, infection with *C. shasta* leads to immunosuppression in the gills of both resistant and susceptible fish, with IFNγ and IFN-stimulated genes being downregulated. However, not all immune genes are downregulated. In resistant fish, upregulation of the cytosolic innate immune receptor NLRC5 occurs in both the gills at 1-day post exposure (dpe) and later in the intestine at 7-dpe. Additionally, two paralogs of *GIMAP4*, a gene involved in T-lymphocyte development, are the most highly upregulated genes in the gills of resistant fish at 1-dpe. The same two paralogs of *GIMAP4* were later found to the most highly upregulated immune genes in the intestine of susceptible fish at 14-dpe, indicating that GIMAP proteins are playing a key role in the host immune response to *C. shasta*.

When the parasite reaches the intestine, susceptible fish fail to control the infection, with no immune genes upregulated and no observable transcriptomic response to the parasite at 7-dpe. This starkly contrasts with resistant fish, which respond with a vigorous IFNγ-driven T_H1 response along with upregulation of MHC Class I genes, *Mx*, and genes involved in antigen processing and presentation. These observations strongly suggest that susceptible fish are failing to recognize *C. shasta* as it migrates to the intestine and its initial proliferation once there. At 14-dpe, susceptible fish have an intense transcriptomic response to the infection, with over 5,000 genes differentially expressed in the intestine. This includes massive upregulation of key immune effectors (IFNγ, IL-1β, IL-6, IL-12, T-bet) that are indicative of a pro-inflammatory T_H1 response. This continues at 21-dpe, where over 12,000 genes are differentially expressed in the intestine. However, this response is ineffective at reducing, or even slowing parasite proliferation and the amount of parasite DNA in their intestines increases exponentially between 7 and 21-dpe. This coincides with the breakdown of their intestinal structure, which is evident in both the histopathology and their transcriptomic response. Genes involved in cell adhesion, cytoskeleton organization, and extracellular matrix formation and adhesion become the most transcriptionally active, indicating that susceptible fish are trying to maintain their tissue integrity as the parasite continues to proliferate in the intestine. At the same time, innate immune receptors that recognize

conserved bacterial motifs, as well as genes encoding bacterial defense proteins become highly upregulated, indicating that their mucosal barrier has become compromised and opportunistic bacteria are beginning to invade the tissue.

The intestinal response of resistant fish is quite different. When exposed to a low dose of *C. shasta*, few if any parasites reach the intestine and the infection is quickly contained. When exposed to a much higher dose, an active infection is established in the intestine and the parasite appears to proliferate at the same rate as in susceptible fish. However, when compared to susceptible fish at the same timepoints, 2-fold less parasite DNA could be detected in intestines of resistant fish, despite receiving a much higher dose of *C. shasta*. Resistant fish also have a much different response to the parasite at the tissue level. The stratum compactum layer of the intestine appears to act as a barrier to parasite migration, limiting its spread to the mucosal surfaces of the intestine, where regeneration of the tissue occurs on a daily basis. This contrasts with what is observed in susceptible fish, where the parasite spreads to all layers of the intestine. This differential response is also evident in the transcriptome of resistant fish. At 7-dpe, there is an over 100-fold increase in expression of caspase-14, which plays a terminal role in keratinocyte differentiation, a type of cell whose primary function is to act as a barrier to biotic and abiotic insults.

The immune response of resistant fish at 7-dpe (NLRC5, IFNγ, Mx, MHC class I) is indicative of a response to an intracellular pathogen. *C. shasta* is considered an extracellular pathogen, and certainly occupies that role in the intestine. However, a previous study has noted a potential intracellular phase for *C. shasta* in the blood vessels early on in the infection. An early intracellular phase of *C. shasta* that is being recognized by resistant fish would explain why we observed upregulation of a cytosolic immune receptor (NLRC5) and why resistant fish have a lower parasite load in their intestine compared to susceptible fish at the same timepoint. An intracellular phase may be required for the development of *C. shasta* in the host, and it may also represent a form of immune evasion that causes the host to initiate a cytotoxic T_H1 response that is ineffective at clearing the extracellular stages in the intestine. This would explain why we observe an exponential increase in the amount of parasite DNA in the intestine of both resistant and susceptible fish between 7- and 21-dpe.

Given how disparate the response of these two phenotypes is early on in the infection, their transcriptomic response in the intestine is remarkably similar late in the infection. Over

7,000 genes are upregulated in both resistant and susceptible fish at 21-dpe, and their expression levels are highly correlated (r = 0.94). Among these shared genes are several key immune factors (IFNγ, IL-1β, IL-6, IL-10) which have previously been implicated in the differences in susceptibility observed between fish stocks. While temporal differences in expression of these genes may be a factor, something that certainly occurs given the delayed immune response of susceptible fish, their expression alone does not explain differences in susceptibility. The primary difference in the immune response of these fish is in the expression of genes related to the adaptive immune response. Resistant fish upregulate significantly more immunoglobulin transcripts and have much higher expression of secreted IgT compared to susceptible fish at the same timepoint. Previous studies have demonstrated that susceptible fish are able to mount an antibody response to *C. shasta*. However, the effectiveness of this response was unclear as it appeared to do little to ameliorate the disease in these fish. It was suggested that this may be due to the response coming too late in the infection, after significant tissue damage has occurred. The results of our study suggest support this hypothesis, as resistant fish were able to limit the extent of tissue damage and mounted an antibody response sooner than their susceptible counterparts.

Genetic mapping of the loci conferring resistance to ceratomyxosis identified a region of chromosome Omy9 that significantly impacted the odds of survival in a backcross generation of rainbow trout. While a different region on Omy11 has a lesser contribution to survival. This result aligns with a previous genetic mapping study conducted on rainbow trout, which identified linkage groups on Omy9 and Omy11 as being associated with resistance to ceratomyxosis. Additionally, a study conducted on resistance to whirling disease in rainbow trout, which is caused by a different myxozoan pathogen, also identified a single region of Omy9 as explaining most of the variation in resistance to the disease. The identification of Omy9 in all three studies, which were conducted on three different rainbow trout stocks, suggest that a conserved mechanism may be at play in rainbow trout, or that Omy9 is enriched for myxozoan disease resistance genes.

Future Directions

This research has identified important gaps in our knowledge and potential avenues for future research. These include: 1) definitively establishing whether *C. shasta* has an intracellular stage in the host, 2) determining the immune mechanism that resistant *O. mykiss* use to resolve

the infection in their intestine, and 3) examining chromosome Omy9 across multiple fish populations and identify regions of genomic divergence between resistant and susceptible fish populations. I would accomplish these aims in the following ways:

1) In order to confirm our hypothesis that *C. shasta* has an intracellular stage in the blood vessels, fish will need to be infected with *C. shasta* and tissue samples corresponding to the migration route of the parasite collected and examined by electron microscopy.

2) To determine how resistant fish resolve the infection, a large group of resistant *O. mykiss* will need to be exposed to high enough dose of *C. shasta* to establish an active infection in their intestine. The fish should be held in 18° C water and intestinal samples collected from six fish each day starting at 21-days post exposure until 40-days post exposure. The samples would undergo DNA extraction to quantify the parasite load and RNA extraction to quantify transcripts related to important T and B cell markers. This would determine if resistant fish switch to a T_H2 response later in the infection, or if they clear it through a different mechanism. Due to the high number of paralogs present in rainbow trout genome, a cost-effective approach that can quantify numerous transcripts at once will need to be employed, such as microfluidic qPCR. An alternative approach that relies on the development of cell surface markers that can accurately distinguish different rainbow trout immune cells, would be to utilize flow cytometry to analyze which cell types are responding the infection.

3) To compare Omy9 across populations, DNA samples would be collected from three phylogenetically distinct populations of resistant *O. mykiss* and three phylogenetically distinct populations of susceptible *O. mykiss*. Deep sequencing of their genomes would be conducted, or if possible, of Omy9 alone. Population genetic statistics (Fst, Tajima's D, π, average heterozygosity) could then be calculated and outlier loci identified that consistently diverge between resistant and susceptible fish populations, which should correspond to the loci for resistance to *C. shasta*. It would then be possible to develop qPCR primers for these regions and examine them in additional individuals or populations.

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