AN ABSTRACT OF THE DISSERTATION OF

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Title: The Ecology of Parasite Interactions within Chinook Salmon.

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Jerri L. Bartholomew

The within-host interactions that can occur as a result of mixed infections in wildlife likely influence the outcome of an infection. We investigated the infection frequency and outcome as well as the potential mechanisms regulating mixed infections with two *Ceratonova shasta* genotypes within the Chinook salmon host. Previous research in our laboratory identified geographic areas throughout the parasite's range where multiple genotypes are sympatric and one area of particular concern where genotypes I and II account for the majority of the total parasites. We used sentinel fish studies to determine the transmission and persistence of both genotypes in Chinook salmon and found that both genotypes were transmitted to the fish host and that a majority (~80%) of initial infections were mixed. To investigate the outcome of mixed infections, we conducted a laboratory study that compared mixed and single genotype infections. Our results demonstrated that the production of mature parasites was reduced in mixed infections when Chinook salmon were exposed to both parasite genotypes simultaneously or genotype I first, perhaps facilitated by a truncated host life span that limited resources for genotype II. Interestingly, an initial exposure to genotype II eliminated competitive suppression and illustrated that prior residency favors the first infecting genotype. Next, using samples collected from the laboratory study, we investigated whether the immune response mediated the mixed infection interaction. The immune response to each genotype alone was specific and when infections were mixed, both genotype-specific

responses occurred. Although the immune response to genotype II was associated with a decrease in genotype II density, the systemic immunosuppression elicited by genotype I prevented host recovery, suggesting the host immune response as one mediator of parasite interactions. Finally, we investigated the use of a less virulent genotype for host immunization against infections with a more virulent genotype. Although, our study demonstrated that immunization was unlikely, a number of study design variations remain to be tested. Taken together, these studies provide evidence for competition between *C. shasta* genotypes that is regulated by the immune response and by limited host resources. Understanding within host dynamics will better inform predictions of genotype re-distributions after barrier removal and fish stock reintroductions as well as parasite virulence evolution.

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The Ecology of Parasite Interactions within Chinook Salmon

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Charlene Nicole Hurst

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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.

Charlene Nicole Hurst, Author

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CONTRIBUTION OF AUTHORS

For the first publication from this dissertation, Peter Wong provided experimental maintenance and sampling assistance as well as sample processing and sequence analysis. Dr. Adam Ray provided advice on the experimental design and statistics as well as samples from both 2008 and 2009. Dr. Sascha Hallett provided oversight for the qPCR assay design and manuscript editing. For the third manuscript from this dissertation, Dr. Brian Dolan provided assistance with cytokine selection and interpretation of the gene expression data.

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CHAPTER 1: INTRODUCTION

WHAT ARE MIXED INFECTIONS AND WHY IS THE STUDY OF THEM IMPORTANT?

Hosts are rarely infected by just one species or strain of pathogen at the same time (Read and Taylor 2001; Telfer et al. 2010), resulting in a mixed infection. Infections of this type allow for interactions to occur among pathogens within the host. Interactions between pathogens are hypothesized to be most severe when the mixed infection is with two strains of the same pathogen species. This is because niche requirements and the host immune response to infection are likely to be more similar between pathogen strains than separate species (Mideo 2009). An interaction (Fig. 1.1) that suppresses pathogen strain transmission and/or abundance is termed competition, while an interaction that increases strain transmission and/or abundance is termed facilitation (Cox 2001). In some cases, these interactions are asymmetric; with one strain affected more than the other. Pathogen interactions in the host may also alter disease severity, disease progression and the host's immune response.

Within an individual host, pathogen interactions (usually competitive) can lead to three infection outcomes that influence pathogen transmission and/or abundance (Fig. 1.1). With co-infection, both pathogens coexist and are transmitted (May and Nowak 1995).With superinfection, a more virulent strain overcomes a less

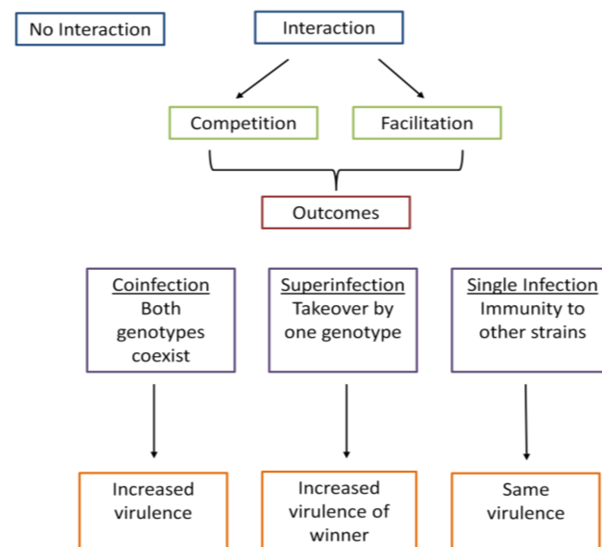

Figure 1.1 Pathogen interaction types and infection outcomes.

virulent strain through competitive exclusion. Thus, only the more virulent strain persists and is transmitted (Levin and Pimentel 1981; Nowak and May 1994; Bremerman and Thieme 1989). In the third outcome, infection results in immunity to other strains. This is essentially a more severe form of the superinfection scenario, where the less virulent strain is prevented from infecting the host.

The order and timing of infection could also influence the dynamics of pathogen strain interactions. The first infecting strain may have an advantage because it can utilize host resources first, perhaps decreasing transmission stage production by subsequent strains (Read and Taylor 2001). Conversely, the first infecting strain may be at a disadvantage if it is the primary target of the immune response or the sole producer of immunosuppressive substances, which requires the use of more parasite resources (Jager and Schjørring 2006; Lello 2012). If these effects are understood, infection order and timing could be manipulated to take advantage of within-host strain dynamics. For instance, hosts exposed to the less virulent strain first may become immunized before infection with the more virulent strain, ultimately preventing or limiting host mortality (Read and Taylor 2001). Thus, the order and timing of infection could eliminate or minimize the effects of an interaction.

During mixed infections, theorists predict that the more virulent pathogen strain will be the better competitor (Bremerman and Thieme 1989; Frank 1996; Mosquera and Adler 1998). This is because high virulence is typically associated with faster within-host growth rates (Frank 1996), allowing the better competitor to produce the majority of the transmission stages. However, the higher virulence associated with a better competitor could be a disadvantage in single infections because of a likely truncated host life span, which limits transmission stage production (Mideo 2009). Therefore, a less virulent strain may have higher fitness in single infections while the more virulent strain has higher fitness during mixed infections (Alizon et al. 2009). The frequency of mixed versus single infections in the system could then determine which pathogen strains dominate at any given time, influencing parasite assemblages within a system.

In addition to the relatively short-term consequences of mixed infections, over time, within-host interactions can lead to selection for certain pathogen strains and

alter the evolution of virulence (Read and Taylor 2001). When a host is infected by a single pathogen, an optimal level of virulence is predicted to evolve, constrained by life history trait tradeoffs (Van Baalen and Sabelis 1995; Bremerman and Thieme 1989). However, the virulence of a pathogen strain could be altered by the continued presence of other strains and lead to evolution of higher virulence if the pathogen is competitively superior and transmits at a higher rate (Levin and Pimental 1981; Bremermann and Pickering 1983; Bremerman and Thieme 1989; Nowak and May 1994; May and Nowak 1995; Van Baalen and Sabelis 1995; Frank 1996).

Predictions pertaining to the evolutionary trajectory depend on the interaction outcome. In both the coinfection and superinfection outcomes, the more virulent strain is the better competitor and average population virulence levels are predicted to increase. The difference in these two infection outcomes occurs in the evolution of virulence at the strain level. When coinfection leads to coexistence, both strains will likely evolve higher virulence to remain competitive (Nowak and May 1994; Mosquera and Adler 1998). In superinfection, frequent mixed infections would favor higher virulence of the more virulent pathogen strain, potentially eliminating the less virulent strain over time. In the extreme case, when infection by one pathogen prevents infection by others, there is no selective pressure to evolve higher virulence (Fig. 1.1; Mosquera and Adler 1998). Thus, although most theories predict that virulence will increase in mixed infections, predictions are likely dependent on the specifics of the system under study.

When other selective pressures are present, such as host availability and host heterogeneity, predictions of virulence evolution become more complicated. For example, under the coinfection outcome, high virulence may be favored if many hosts are available, while low virulence may be favored if hosts are rare, as more transmission stages could be produced in the extended amount of time till host death (Nowak and May 1994). In a heterogenous host population, virulence evolution also depends on trait tradeoffs that are required for success in different host species or in hosts with varying pathogen susceptibilities (Regoes et al. 2000; Alizon and van Baalen 2008).

HOW COMMON ARE MIXED PATHOGEN STRAIN INFECTIONS?

A number of studies have focused on understanding within-host pathogen strain interactions in a variety of pathogen systems. These include the protozoans *Plasmodium chabaudi* (de Roode et al. 2005a, b) and *Trypanosoma brucei* (Balmer et al. 2009), the trematode *Schistosoma mansoni* (Davies et al. 2002; Gower and Webster et al. 2005), the bacterium *Pasteuria ramosa* (Ben-Ami et al. 2008), the virus *Infectious Hematopoietic Necrosis Virus* (IHNV; Wargo et al. 2010; Paneranda et al. 2011) and the fungus *Metarhizium anisopliae* (Staves and Knell 2010).

The first step in understanding the ecological role of within-host pathogen strain interactions is to determine how frequently mixed infections occur within the host population. Both *P. chabaudi* and *T. brucei* lead to diseases in mice that are similar to the human manifestations of malaria and African sleeping sickness, respectively. Thus, understanding the outcome of mixed-strain infections with these pathogens in natural populations is likely to be important, even if the prevalence is low because of the implications for human health. Malaria can be caused by a variety of *Plasmodium* species in humans with the prevalence of mixed strain infections ranging from 5.6 to 83%, depending on the geographic location surveyed. *T. brucei* mixed strain infection prevalence was typically lower in vertebrate hosts (approximately 9%) than it was in the tsetse fly vector (42.9%; Balmer and Tanner 2011). Although *S. mansoni* also causes a human disease, schistosomiasis, much of the research on mixed-strain infections focuses on the intermediate snail host. In the areas snails were surveyed, the prevalence of mixed-strain infections was 54.1% in Brazil and 11.6% in Guadalupe (Balmer and Tanner 2011).

For the pathogens remaining from the above list, information on mixed infections in natural populations is limited, likely because they have no direct impact on human health and/or are adapted for laboratory use. For both *P. ramosa* and *M. anisopliae,* information on the prevalence of mixed strain infections in natural host populations is lacking. In the case of IHNV, this virus can be subdivided into three major genogroups; U, M and L. Genogroup M is most closely associated with rainbow trout farms in Idaho and several strains (MA-MF) are known to have co-circulated throughout the area over the last 20 years (Troyer et al. 2008). Therefore,

fish from Idaho are likely to be infected with multiple IHNV strains within genogroup M, leading to a high prevalence of mixed infections.

WHAT INTERACTIONS TAKE PLACE DURING EMPIRICAL STUDIES?

A. *Plasmodium chabaudi* in mice (Fig. 1.2; de Roode et al. 2005a, b)

Using two strains of *P. chabaudi* that differed in their virulence in mice, the more virulent parasite strain competitively suppressed the less virulent strain resulting in a decrease in density of the less virulent strain when exposure to both strains occurred concurrently. This suppression led to a decrease in the transmission of the less virulent parasite strain to the mosquito host. Interestingly, when the less virulent strain was allowed to infect first, competition was eliminated and production by both strains was the same as in single infections. When the more virulent strain infected first, the less virulent strain was suppressed. The severity of the suppression increased as the length of time between exposures increased.

B. *Trypanosoma brucei* in mice (Balmer et al. 2009)

This study investigating mixed parasite strain infections assessed potential interactions between two trypanosome strains with relatively equal virulence and replication rates, and two strains with differing virulence, where the less virulent strain replicated more slowly. Parasite density assessed a few days post infection indicated that both strains were equally competitively suppressed when virulence between the strains was similar compared to single infections with either strain. However, when virulence differed, suppression was more severe for the less virulent strain. Surprisingly, host survival was increased by about 15% when the mixed infection included both strains of differing virulence, suggesting that the less virulent strain may benefit the host in terms of survival.

C. *Schistosoma mansoni* in the intermediate snail host (*Biomphalaria glabrata*; Davies et al. 2002; Gower and Webster 2005)

The results of the two *S. mansoni* studies may at first appear to be at odds; however, Davies et al. (2002) performed their experiments with parasite strains of similar virulence, while Gower and Webster (2005) used parasite strains that differed in virulence. Davies et al. (2002) demonstrated that parasite replication was increased

during the earlier stage of infection, but overall parasite production did not differ between single and mixed infections because of a faster host mortality rate in snails with mixed infections. This faster mortality was also associated with decreased reproductive success for the host. Therefore, it is difficult to ascertain which interaction actually took place between parasite strains with similar virulence, although the initial increase in parasite replication suggests facilitation. The Gower and Webster (2005) study differed from the other examples in that the less virulent parasite strain was the better competitor both in simultaneous and sequential infections, as evidenced by suppression of only the more virulent strain. This could be explained by the faster replication rate of the less virulent strain.

D. *Pasteuria ramosa* in *Daphnia magna* (Ben-Ami et al. 2008)

Once again, in this system we see evidence for competitive suppression of the less virulent pathogen strain through a reduction in transmission stage production. This occurred when both pathogen strains were introduced simultaneously and when the more virulent strain was first to infect. However, when the less virulent strain infected first, it produced a larger proportion of the transmission stages compared with the other mixed infection scenarios, providing more support that the first infecting strain has a competitive advantage. As observed with *T. brucei*, the host benefited from the mixed infection as the fecundity costs typically seen in single infections (castration before any eggs are laid), were delayed.

E. *Infectious Hematopoietic Necrosis Virus* (IHNV) in rainbow trout and sockeye salmon (Wargo et al. 2010; Paneranda et al. 2011)

Studies with IHNV have examined potential interactions between and within genogroups. Consistent with theory, the more virulent genogroup or strain within a genogroup consistently produced the highest viral titre. However, there was no evidence of an interaction between genogroups or strains within a genogroup as viral titre remained unchanged compared with single infections.

F. *Metarhizium anisopliae* in wax moths (*Galleria mellonella*; Staves and Knell 2010).

M. anisopliae is a fungus that requires host death before sporulation. The authors examined potential interactions between two strains of fungus with differing

virulence and found that the more virulent strain did competitively suppress conidia production of the less virulent strain during mixed infections and was the superior competitor.

These studies demonstrate that when mixed infections with multiple strains of the same pathogen are present in a single host, competitive interactions between pathogen strains are likely to occur, although there are exceptions (e.g. IHNV). In addition, whether the strains are relatively similar in virulence or differ should be considered when formulating predictions about the interaction type and outcome. When strains differ in virulence, the strain that is most likely to be the better competitor is typically the more virulent strain because high virulence is often associated with traits that allow for faster host exploitation, such as an increased within-host growth. From the few studies that also examined sequential infections, the first infecting strain does typically have a competitive advantage. Thus, the systems that have examined pathogen strain interactions do largely support the assumptions of competition theory.

	P. chabaudi		*T. brucei*			*S. mansoni*			*P. ramosa*		*M. anisopliae*		*IHNV*	
Strain Virulence	more	less	more	less	same	more	less	same	more	less	more	less	more	less
Concurrent	No change	↓	↓	↓	↓↓	↓	No change	↑↑ ?	No change	↓	↓	↓	No change	No change
Sequential-Less 1st	No change	No change	NE			↓	No change	NE	No change	No change	NE		NE	
Sequential-More 1st	No change	↓				↓	0		No change	↓				

Figure 1.2 Summary of interactions across multiple pathogen-strain systems. Downward arrows indicate competitive suppression, upward arrows indicate facilitation. Smaller arrows indicate less severe competitive suppression. NE indicates where experiments have not been conducted to investigate sequential infections.

WHAT MECHNISMS GOVERN PATHOGEN STRAIN INTERACTIONS?

Within host interactions are mediated either directly or indirectly (Fig. 1.3) and understanding how this is achieved may aid in the control of disease. Direct interactions occur through interference (encounter), by using chemical substances or through mechanical facilitation. For example, *Streptococcus pnemoniae* produces the toxin hydrogen peroxide which kills the bacterium *Staphylococcus aureum*. However,

toxin production can negatively affect the producer if resources are drawn away from other life functions, such as growth, to produce the toxin (Selva et al. 2009). In mechanical facilitation, infection with one pathogen may facilitate infection with another by compromising host tissues and increasing host susceptibility to secondary infections. Evidence for this was observed in rainbow trout where infection with the parasite *Argulus coregoni* increased the susceptibility of the fish host to subsequent infection with the bacterium *Flavobacterium columnare* (Bandilla et al. 2006).

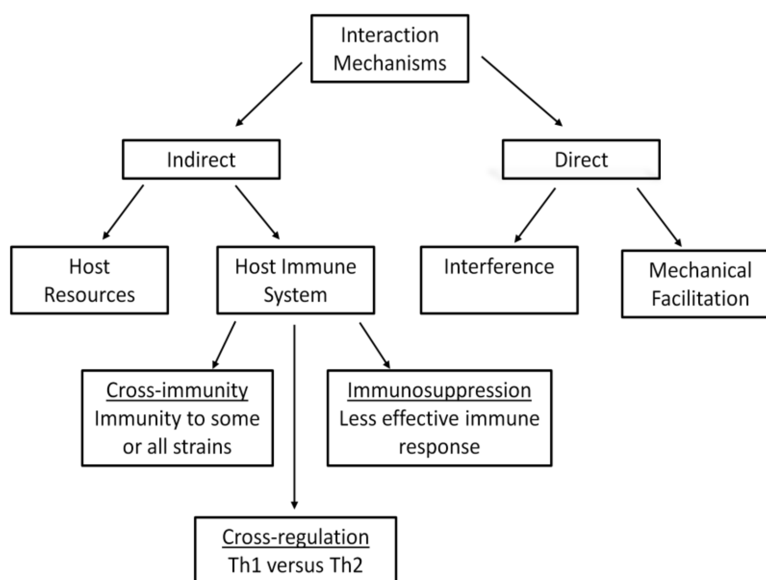

Figure 1.3 Mechanisms mediating pathogen strain interactions.

Indirect interaction mechanisms include resource limitations and the host immune response (Fig. 1.3). Resource limitation can lead to the partitioning of resources or adaptation to become better able to acquire those resources before a competitor (Mideo 2009). For example, in malarial infections, researchers hypothesized that the availability of red blood cells, required by the intracellular parasite, mediated the competitive interactions between malarial strains (Bell et al. 2006). The immune response mechanism can be divided into three subcategories; cross immunity, cross-regulation and immunosuppression. In cross immunity, the immune system suppresses all infecting pathogens (Mideo 2009). During cross-regulation, the host immune response targets pathogens with either the T-helper cell 1 (Th1) or T-helper cell 2 (Th2) responses. If the Th1 response is mounted in response

to an intracellular pathogen, like the causative agent of tuberculosis, than the Th2 response cannot be mounted to fight a helminth infection (Ezenwa et al. 2011). Lastly, immunosuppression may facilitate further infections by other pathogen species/strains by decreasing the effectiveness of the immune response (Mideo 2009).

Deciphering the mechanism(s) regulating pathogen interactions is complicated by a need to separate host and pathogen effects. While indirectly competing for host resources seems to be the most commonly hypothesized mediator of pathogen interactions (Staves and Knell 2010; Gower and Webster 2005), immune mediated competition (Balmer et al. 2009) and direct interference (Gower and Webster 2005) have also been hypothesized to mediate interactions in some systems. Even in what appears to be the best studied system, mice and the causative agent of malaria (*P. chabaudi*), answers are not straightforward. Both limited resources as well as the host immune response were implicated as potential mechanisms in interaction studies (de Roode et al. 2005a, b). Testing for the role of the adaptive immune response in regulating parasite interactions within the host required the use of mice deficient in an adaptive immune response. The authors demonstrated that competition was alleviated later in the infection in mice without an adaptive immune response, but competition still occurred before the adaptive response would even be mounted. This led to the hypothesis that another mechanism, likely resource competition, mediates the early interaction (Råberg et al. 2006).

Although the Råberg et al. (2006) study serves as a model for how to answer the question of immune-mediated competition in pathogen systems that use a mouse host, the ability to use immunodeficient hosts does not exist in many other host-pathogen systems. Even though understanding the mechanism(s) responsible for regulating interactions is important for predicting infection outcomes and developing disease control programs, the technical difficulty associated with these studies means that few studies have been able to examine pathogen interaction mechanisms.

CERATONOVA SHASTA (SYN. CERATOMYXA SHASTA) EXPERIMENTAL SYSTEM

Ceratonova shasta (Atkinson et al. 2014) is a myxozoan parasite, which requires an invertebrate and vertebrate host to complete its lifecycle. The waterbourne myxospore stage is ingested by the polychaete, *Manayunkia speciosa* and develops within the polychaete into an actinospore, which is released into the water column. Salmon or trout become infected with the actinospore stage after it attaches to and penetrates the gill tissue. Parasite proliferation begins in the gills and continues as pre-spore stages travel though the blood vessels to the target tissue, the intestine. Once the parasites reach the intestine, they continue to develop into the mature myxospore stage. Infections can result in the disease enteronecrosis, which is fatal due to hemorrhaging and intestinal necrosis (Bartholomew et al. 1997; Bjork and Bartholomew 2010). Whether or not a host succumbs to infection with a particular genotype is partly dependent on host resistance to the pathogen. Fish originating in waters where *C. shasta* is endemic have developed some inherent genetic resistance to the parasite (Bartholomew 1998).

Although found in freshwater from Alaska to northern California, a large portion of research on *C. shasta* has concentrated on understanding its dynamics in the Klamath River, Oregon-California. This focus is due to the recent declines in adult salmonid populations attributed to the high mortality of juvenile salmonids from *C. shasta* (Fujiwara et al. 2011). It was also in the Klamath system that the four *C. shasta* genotypes, 0, I, II and III were first elucidated. Using the Internal Transcribed Spacer Region I of the rRNA operon, the four genotypes were genetically distinguished by the number of trinucleotide repeats (Atkinson and Bartholomew 2010a, b).

Previous studies have shown that each genotype was associated with infection and disease in a unique subset of salmonid hosts. Genotype I was most commonly isolated from Chinook salmon (*Oncorhynchus tshawytscha*) and was the only genotype associated with mortality of this host species, although infections with genotype II did occur. Genotype II was associated with infection and mortality of both coho salmon (*O. kisutch*) and rainbow trout (*O. mykiss*), while genotype 0 was associated with non-lethal infections in steelhead/redband trout (*O. mykiss*; Atkinson and Bartholomew 2010a, b; Hurst and Bartholomew 2012). Although genotype III is

usually only present in the river in minor amounts, it appears capable of infecting all species sampled. Subsequent surveys of *C. shasta* in other river systems including the Deschutes and Willamette in Oregon, the Cowlitz in Washington and the Sacramento in California (Stinson 2012), have demonstrated conservation of these genotypes and their host associations throughout the parasite's range.

The presence of multiple parasite genotypes sympatric with the fish host has created an environment in which mixed infections are likely to frequently occur. Mixed infections in the fish host then allow for potential parasite genotype-genotype interactions. Ultimately, the occurrence of an interaction could influence the infection outcome in an individual host and aid in our understanding of disease dynamics in river systems with *C. shasta*.

DISSERTATION HYPOTHESES

The chapters in this dissertation address the following hypotheses:

Chapter 2

H_1**:** *C. shasta* genotypes are not selectively transmitted, resulting in mixed initial infections.

Chapter 3

H_1**:** *C. shasta* genotypes compete during concurrent mixed infections.

H_2**:** The more virulent genotype will be the better competitor.

H_3**:** In mixed sequential infections, the first infecting genotype has a competitive advantage over the second.

Chapter 4

H_1**:** The host immune response is parasite genotype-specific.

H_2**:** During coinfection, the immune system responds to both genotypes and renders the immune response ineffective.

Chapter 5

H_1**:** Exposure to a less virulent genotype (II) prior to a more virulent genotype (I) reduces host disease severity/myxospore production.

CHAPTER 2: TRANSMISSION AND PERSISTENCE OF *CERATOMYXA SHASTA* GENOTYPES IN CHINOOK SALMON

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ABSTRACT

Ceratomyxa shasta is a myxozoan parasite of salmon and trout transmitted by waterborne actinospores. Based on DNA sequence data and host specificity, four distinct parasite genotypes are recognized. Genotypes I and II are common in the lower reaches of the Klamath River, OR-CA, but only infection by genotype I causes mortality in Chinook salmon. We conducted sentinel fish exposures and determined genotype composition in river water during exposure, and in fish gills, intestine and tank water post-exposure to determine if: 1) transmission of parasites having different genotypes is host-specific and 2) all transmitted genotypes persist in the host through to release as waterborne stages. Initial parasite transmission to the fish host appears indiscriminant as we detected both genotypes I and II in 83.6% of the fish gills sampled. However, only genotype I was detected in fish that succumbed to infection, while both genotypes persisted in fish that survived. Persistence was likely dependent on exposure dose, initial infection type (mixed or single) and infection outcome (mortality or survival). The transmission of both genotypes to a majority of Chinook salmon and the persistence of multiple genotypes raises questions about how infection with mixed genotypes could result in within-host interactions that affect disease severity.

INTRODUCTION

The myxozoan parasite *Ceratomyxa shasta* has been isolated from a variety of salmonids throughout the Pacific Northwest (Hendrickson et al. 1989; Bartholomew 1998). Infected fishes release myxospores into the water column and these must infect the polychaete host, *Manayunkia speciosa* (Bartholomew et al. 1997) to complete the life cycle. Actinospores released from the polychaete attach to and penetrate the gill epithelium of the fish, proliferate in the blood and migrate to the intestinal tissues where myxospore development occurs (Bjork and Bartholomew, 2010).

In the Klamath River, Oregon-California, *C. shasta* densities can reach >100 spores/L of river water during late spring through early summer (Hallett et al. 2012). Infections at these high densities have led to high mortality in juvenile salmonids and declines in returning adult populations (Stocking et al. 2006; Nichols et al. 2009; Fujiwara et al. 2011; Hallett et al. 2012). Recently, four distinct genotypes of *C. shasta,* based on Internal Transcribed Spacer Region 1 (ITS1) sequences, were identified in fishes and water collected from the Klamath River (Atkinson and Bartholomew 2010a, b). The concurrent presence of these four genotypes creates potential for mixed pathogen infections in salmonid hosts.

Mixed pathogen infections are common in wildlife and can lead to changes in host mortality, pathogen production and/or pathogen persistence (Read and Taylor 2001; Oh et al. 2006; Telfer et al. 2010). In the Klamath system, the dominant *C. shasta* genotypes are I and II, which cause mortality of Chinook (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*), respectively. Lesser amounts of genotype 0, found in steelhead and redband trout (*O. mykiss*), and genotype III, isolated from multiple hosts, are detected (Atkinson and Bartholomew 2010a, b). Although multiple genotypes occur simultaneously in the water column, typically only a single genotype is detected in a fish that succumbs to infection (Atkinson and Bartholomew 2010a, b; Hurst and Bartholomew 2012; Stinson 2012). In contrast, multiple genotypes are often present in fish that survive exposure (Atkinson and Bartholomew 2010b) and in fish sampled prior to mortality (Stinson 2012). Because these studies did not examine genotype composition at the site of attachment or early in the infection, it is unknown

if fish were infected with only one genotype or if within host processes (e.g. host immune response, parasite interactions) influenced genotype composition.

Detection of multiple genotypes in some infected fish led us to hypothesize that *C. shasta* genotypes are not selectively transmitted and that initial infections will be mixed. We also hypothesize that mixed infections will persist in fish that survive, which typically occurs at low exposure doses. To address these hypotheses, we exposed Chinook salmon to the parasite and assessed genotype composition in: river water during exposure, gills (to assess parasite genotype transmission), intestine (to assess parasite genotype persistence), and in the water column prior to host death (to assess parasite genotype release). Understanding the outcome of mixed infections and whether multiple pathogen genotypes can persist in a single host is important for predicting disease dynamics in a system. For example, in the Klamath River, Chinook salmon are the dominant salmonid and could act as a reservoir host for genotype II, the lethal genotype for threatened coho salmon populations.

MATERIALS AND METHODS

Fish exposure and sample collection

In addition to the field exposure conducted in June 2012, we used archived fish samples from previous field exposures (June 2008, 2009), to test our hypotheses. For all exposures, Chinook salmon (age-0, 5-10 g) from Iron Gate Hatchery (Hornbrook, CA) were held in cages in the Klamath River at a site with high spore densities (Beaver Creek, 41°52.1'N, 122° 48.6'W). At the beginning and end of each exposure, 3 1-L water samples were collected and filtered within 24 hr to determine spore density and genotype composition during exposure.

Ninety Chinook salmon were divided into three cages of 30 fish each and exposed for 24 hr in June 2012. An additional 50 fish were reserved as un-exposed controls with 25 fish in each of two replicates. After the 24 hr exposure, 10 fish in each treatment replicate and five fish in each control replicate were euthanized with an overdose of tricane methansulfonate (MS-222, Argent Laboratories, Redmond, WA, USA). To assess transmission, the entire gill from the left side of the head was collected and frozen at -20°C. Gill tissue will include actinospores attached to gill

epithelium, invading stages and proliferating blood stages (Bjork and Bartholomew 2010).

The remaining fish were transported to the Oregon State University-John L. Fryer Salmon Disease Laboratory (SDL) in Corvallis, OR and held in 25-L tanks (one cage per tank) containing flow-through, specific pathogen-free well water. Fish were treated immediately after exposure for potential bacterial infections by feeding a TM100-medicated diet (Bio-Oregon) and for external parasites by bathing for 1 hr in 125–170 mg/L of formalin for three consecutive days (Stocking et al. 2006). Fish were then fed and monitored for clinical disease signs daily. To assess persistence, all moribund fish and those surviving until the end of the study at 60 days were euthanized and a 25 mg portion of the intestine was collected from each fish and frozen at -20 °C. To assess release, tank water was collected beginning at14 days post-exposure and continued twice weekly until 45 days post-exposure. Prior to water sampling, inflow to each tank was stopped and the tanks were aerated for 24 hr, followed by collection of three 1-L water samples.

To further support the hypothesis that both parasite genotypes are transmitted, we determined genotype composition in archived gill and intestinal samples from a study conducted in June 2009 when parasite dose was low that investigated parasite transmission under differing flow regimes (Ray and Bartholomew 2013). In that study, three replicates of 25 fish were exposed for 6 hr, then 10 fish from each replicate were immediately euthanized and gill samples were collected as above and stored in 95% ethanol. All moribund fish and those surviving until the end of the study at 60 days were euthanized; intestines were collected, stored as above and were used to assess genotype persistence.

To examine genotype persistence when a high dose results in mortality, we determined genotype composition in archived intestinal tissue and tank water samples from a study conducted in June 2008 that established a mortality threshold for *C. shasta* (Ray et al. 2010). In that study, four cages, each containing 40 Chinook salmon, were exposed for 72 hr. Intestines from a subset of 10 moribund fish from each cage were used to assess persistence as above. Two liters of water were collected from each tank at 17 days post-exposure as described above.

Sample processing

DNA from water samples was extracted and purified with a commercial kit (Hallett and Bartholomew 2006). DNA from the intestine and gill was extracted by adding 495 µL tissue lysis buffer (ATL, Qiagen) and 5 µL of Proteinase K (Qiagen) to the sample followed by incubation at 37°C overnight on a rocking platform. The following day, 100ug/mL of RNAase A was added and samples were incubated at 37°C for an additional hour and then boiled for five minutes (Palenzuela et al. 1999). For gill samples, a 60 µL aliquot of the homogenous lysed tissue solution was purified using the Qiagen DNeasy Blood and Tissue kit® (Valencia, CA, USA) and eluted into 60 µL of buffer AE.

Molecular analyses

Total parasite density in the river water and tank water samples was determined using an established *C. shasta*-specific qPCR (ssurRNA gene; Hallett and Bartholomew 2006). Each sample was assayed in duplicate and the Cq (quantitative cycle) values averaged. A sample was considered positive if both wells fluoresced. A sample was re-assayed if duplicate wells differed by >1 standard deviation and at least one well corresponded to 1 spore L^{-1}. Each plate included two negative controls (molecular grade water) and two positive controls in duplicate. In addition, one water sample replicate per collection was assessed for inhibition with a separate IPC-qPCR assay (Hallett et al. 2012). After processing, the Cq values for each sample were averaged to yield one value per tank or exposure and converted to number of spores/L using a standard curve (Hallett and Bartholomew 2006). Total parasite dose was calculated according to Ray and Bartholomew (2013) and incorporated water velocity, spore density and exposure duration.

A third qPCR assay was developed to amplify parasite ITS1-DNA from water, gill and intestine samples for Sanger sequencing of the ITS1 gene to determine genotype composition. The rRNA gene arrays of all known *C. shasta* ITS1 genotypes (0, I, II, III and respective subgenotypes; Atkinson and Bartholomew 2010a, b) were aligned in BioEdit (Hall 1999) and novel primers were designed manually that spanned the variable ITS1 region. Primer specificity was assessed *in silico* using BLAST (http://blast.ncbi.nlm.nih.gov/) and combinations of three forward and three

reverse primers (Invitrogen, Life Technologies) were assessed *in vitro*. The chosen pair, forward primer CsgenF4 5' GGCAGAATTTATTTTGTCG 3' and reverse primer CsgenR1 5'AGGGATCCACCGTTAAC 3' amplified 161 bp; *Myxobolus cerebralis*, *Ceratonova gasterostei* and *Parvicapsula minibicornis* did not amplify. A sample subset was compared with the previous ITS1 genotyping assay (Hallett et al. 2012).

The optimized reaction and cycle conditions follow for the ITS1-qPCR assay. Each 20 µL reaction contained: 10 µL of stock TaqMan® Gene Expression Master Mix (ABI, Life Technologies), 4.7 µL of molecular grade water, 1.6 µL each of 10 µM forward and reverse primer, 0.5 µL of 10 mg/mL BSA, 0.6 µL of 1.5 µM SYTO-9 green fluorescent nucleic acid stain (Invitrogen, Life Technologies, Grand Island, NY) and 1 µL of extracted DNA sample. The use of an intercalating-dye-based qPCR rather than a probe-based qPCR allowed us to capture all possible genotypes (at least four). We also used SYTO9 in lieu of the standard SYBR Green dye because the latter has demonstrated dye concentration and DNA concentration issues (Monis et al. 2005). Reactions were run in ABI Prism® 96-well optical reaction plates (Foster City, CA, USA) on an ABI 7300 Real Time PCR System. All tissue and samples were run singly on a plate, akin to PCR, along with a negative (with molecular grade water) and positive control (with parasite DNA). The cycling conditions were: 2 min hold at 50°C, 10 min hold at 95°C followed by 40 cycles of 95°C for 15 sec and 58°C for 1 min 30 sec. A dissociation step was added to allow for visualization of the product/s: 95°C for 15 sec, 60°C for 1 min, 95°C for 15 sec and 60°C for 15 sec. A FAM reporter was selected as per Monis et al. (2005), no quencher was used and the passive reference was ROX.

Samples with dissociation curves between 72-73°C were diluted 1:10 with molecular grade water and submitted for Sanger sequencing with CsgenF4 at the Center for Genomics and Bioinformatics at Oregon State University, Corvallis, OR. Parasite genotypes were differentiated and proportions were assessed according to Atkinson and Bartholomew (2010a). To assess apportioning of genotypes post-amplification versus actual composition, we prepared samples with known quantities

of genotype I and II with the following ratios: 9:1, 7.5:2.5, 5:5, 2.5:7.5 and 1:9, amplified each sample in triplicate then sequenced each product.

Statistical Analysis

To determine if genotype I proportions in individual gills differed from the mean proportion in river water in 2009 and 2012, we used a one sample t-test in R version 2.14 ($P < 0.05$ was considered significant).

RESULTS

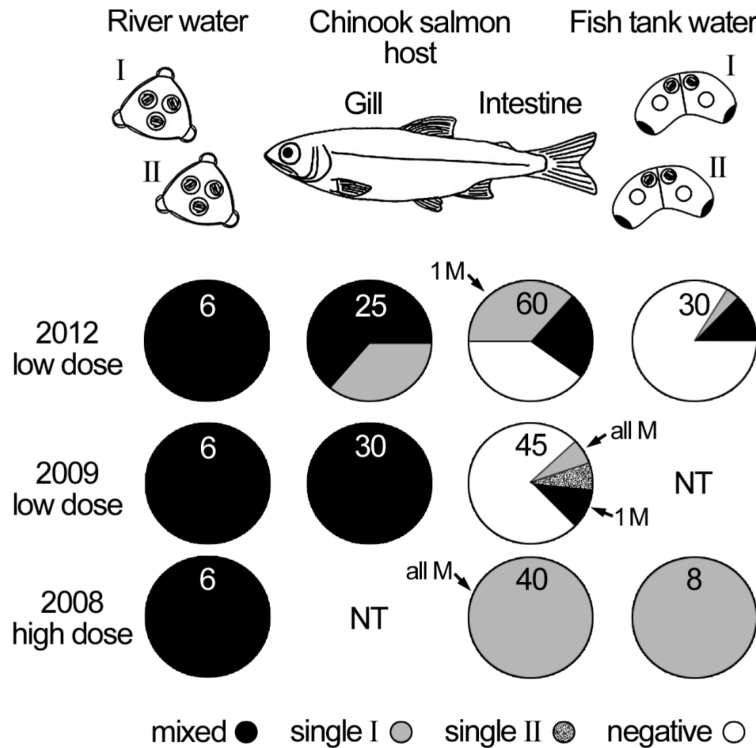

Figure 2.1 Transmission and persistence of *Ceratomyxa shasta* genotypes I and II in river water, Chinook salmon gills, intestines (M indicates mortalities) and holding tank water during the three study years. NT refers to samples that were not collected. Relative parasite exposure doses appear below each year. River water was collected at the field exposure site (Klamath River main-stem above the confluence with Beaver Creek). The numbers contained within each pie chart represent the sample size. In the legend, mixed indicates samples with a combination of genotypes I and II and single indicates detection of only genotype I or II. Negative samples indicate no parasite was detected.

Parasite genotype composition in water

Water samples from all years were a mix of genotypes I and II, with genotype I constituting the majority in all samples (Fig. 2.1). In 2012, exposure dose was 7.7 x 10^4 spores fish^{-1} (4.9 spores L^{-1} ± 3.1 SE) and consisted of 78% I and 22% II (± 1 SE). Exposure dose and genotype proportions in 2009 were similar; 6.8 x 10^4 spores fish^{-1} (25.6 spores L^{-1} ± 16.2 SE) comprised of 75% I and 25% II (± 1 SE; Ray and Bartholomew 2013). In 2008, the exposure dose was higher and genotype proportions differed from previous years; 1.3 x 10^7 spores fish^{-1} (477.3 spores L^{-1}; Ray et al. 2010) consisted of 63% I and 37% II (± 1 SE).

Transmission of parasite genotypes

Prevalence of infection (detection of parasite DNA) in fish gills was 100% in the two years this tissue was sampled, despite low parasite doses. In 2012, mixed infections occurred in 64% (16/25) of the samples with an average of 84% I and 16% II (± 1 SE). The remaining 36% (9/25) were infected with genotype I only (Fig. 2.1). Five additional samples yielded a product, but the sequences of these were unreadable and thus excluded from further analyses. In 2009, all infections were mixed and mean genotype proportions were the same as in 2012: 84% I and 16% II (± 1 SE). The proportion of genotype I was significantly higher in the gills than the river water in both 2012 and 2009 ($t_{24} = 4.0$, $p < 0.001$; $t_{29} = 11.2$, $p < 0.001$, respectively).

Persistence of genotypes in the intestine

When Chinook salmon survived *C. shasta* infection both genotypes were able to persist, but in fish that succumbed to infection typically only genotype I was detected. In 2012, only genotype I was detected in the single fish that succumbed to disease. Of the 58 challenge survivors, 13 had mixed infections (82% I, 18% II, ± 1 SE), 20 had single infections with genotype I, and 25 were negative. In 2009, four fish died from infection (9%, Ray and Bartholomew 2013); three fish with genotype I only and the fourth with a mixed infection of 87% I and 13% II. Of the 41 fish that survived the challenge, four had mixed infections comprised of 75% I and 25% II (± 4 SE), three had single infections with genotype II and 34 were negative. In 2008, 95% (57/60) of the fish succumbed to infection (Ray et al. 2010). The entire subset of these fish examined in this study (40/60) had single infections with genotype I (Fig. 2.1). No

parasite was detected in the intestine of any unexposed controls.

Parasite release from salmon host

In 2012, parasite DNA was detected in the water of the holding tank in quantities above 1 spore L^{-1} in only one of the three replicates from 24 to 38 days post-exposure, with a mean density of 7.2 spores L^{-1} (± 1.3 SE). Except for the sample collected on day 24, in which only genotype I was detected, all positive samples contained a mixture of genotypes with a mean of 86% I and 14% II (± 1 SE). In 2008, when all fish succumbed to infection, only genotype I was detected in the water sampled at day 17 post-exposure (Fig. 2.1). Parasite density in these water samples was 211.0 spores L^{-1} (± 122.1 SE).

Genotype assay

We found that inhibition was less often an issue with the ITS1-qPCR assay compared to the PCR of Hallett et al. (2012) for genotyping gill samples likely because of the smaller amplicon size as compared to PCR. We were unable to sequence gill samples from 2009 using PCR, but a majority of the samples could be sequenced using the ITS1-qPCR. The variation among genotype proportions of a sample assayed in triplicate ranged from 1 to 5%. A linear relationship among combinations of genotype proportions was confirmed, although a mean genotype bias of 9% was observed for genotype II (data not shown). This would result in overestimation of genotype II in our samples and suggests that if genotype II was present it would have been detected by our assay. Logistically, the ITS1 assay also decreased sample processing time because sufficient amplification occurred in one round of qPCR as opposed to two rounds of PCR and purification of amplicons for sequencing was no longer necessary.

DISCUSSION

Ceratomyxa shasta dynamics in a river system are likely influenced by the outcome of mixed genotype infections. In the Klamath River, both *C. shasta* genotypes I and II were detected in the river and the majority of the initial infections in Chinook salmon were mixed (83.6%), supporting our hypothesis that genotype transmission is not selective. The proportion of genotype I was higher in the gill compared to the river at the time of exposure, which could be explained by more rapid replication of genotype

I after transmission (author's unpublished data), indicative of invading parasites. Although genotype I caused lethal infections, both genotypes were detected in fish that survived mixed infections. This supports our second hypothesis that both genotypes can persist in Chinook salmon. Assay of water from the holding tanks demonstrated that both parasite genotypes were released from the fish host prior to death. However, because we were unable to identify the developmental stage released, it is unknown if these were mature myxospores that could successfully transmit to the polychaete host. Thus, although exposure of Chinook salmon to multiple genotypes may result in mixed genotype infections, genotype persistence is associated with infection outcome (morbidity or survival), exposure dose (high or low) and initial infection type (mixed or single).

Myxozoans display a broad range of host specificity with some species such as *Myxobolus cerebralis* and *C. shasta* infecting only salmon and trout, whereas *Kudoa thyrsites* and *Enteromyxum leei* parasitize a variety of fish families (Lom and Dyková 2006). The ability to select preferentially a more susceptible host species has been demonstrated for a few myxozoans (Yokoyama et al. 1997; Kallert et al. 2005; Yokoyama et al. 2006). For example, *Thelohanellus hovorkai,* which infects common carp, did not invade goldfish (Yokoyama et al. 2006). However, this finding is not universal, as *M. cerebralis* does not discriminate among fish species and/or strains at the entry site. After exposure to the same parasite dose, there was no difference in the numbers of *M. cerebralis* in the epithelium of susceptible and resistant strains of rainbow trout (Kallert et al. 2009). Similarly, there was no difference in the number of *C. shasta* genotype I spores transmitted to the gills of both resistant and susceptible Chinook salmon hosts (Bjork et al. 2014). Our study of *C. shasta* genotype transmission corroborates the findings of other myxozoan systems that selection at the site of attachment does not occur.

The persistence of genotypes in mixed infections is likely related to exposure dose and survivorship. Survivorship of Chinook salmon is low when the exposure dose is greater than 7.5×10^4 total spores per fish, or exceeds a density of genotype I greater than 10 spores per L in the river (Ray et al. 2010; Hallett et al. 2012). Providing further support for these thresholds, when both the total dose and genotype

I density thresholds were surpassed (in 2008), mortality in Chinook salmon was high and only genotype I was detected. Although gill samples were not collected, we assume that most fish were initially infected with both genotypes as both were detected in the river at exposure. When only one threshold was exceeded, as in 2009 (surpassed genotype I density only) and 2012 (surpassed total dose only), survival was high and both genotypes persisted to release. Therefore, it appears that in addition to survival, both the genotype I density and total parasite dose thresholds could be used for predicting genotype persistence.

The persistence of genotype I in Chinook salmon is influenced by whether initial infection is with a mixture of genotypes (I and II) or with genotype I only. When we compared the persistence of mixed and single genotype I infections in 2012, the proportion of fish with genotype I only remained consistent throughout the infection (36% in gills, 33% in intestine). However, the proportion of fish that retained mixed infections to the end of the study decreased by about 40%, indicative of recovery. Clearance of both genotypes from initially mixed infections also occurred in 2009, with 75% of the survivors negative for the parasite. Thus, the persistence of genotype I was reduced in mixed infections compared with single genotype I infections. This suggests that co-infection with genotype II may facilitate recovery from genotype I infections.

Because Chinook salmon are semelparous and die after spawning, it is thought the parasite life cycle has evolved to take advantage of this natural dispersal mechanism, with parasite release occurring after the death of the salmon host on the spawning grounds (Hallett and Bartholomew 2012). Our study supports this hypothesis, as densities > 1 spore L^{-1} were detected only from tanks where mortality eventually occurred. Thus, diseased fish likely disseminate parasites along their migration route. However, it is unknown if the parasite stages released prior to host death are capable of maturing and infecting the polychaete host.

Within-host processes, such as direct genotype interactions or indirect regulation by the host-immune response, likely influence infection outcomes. These interactions can affect both the host and the parasite by reducing genotype density or persistence within the host and/or increasing disease severity (Read and Taylor 2001).

Conversely, parasite density and persistence may be increased after suppression of the host immune system (Telfer et al. 2010). In some cases, the host immune response may preferentially target one genotype over another, as evidenced in infections with different strains of *Plasmodium falciparum* in mice (Råberg et al. 2006). In our study, persistence of both genotypes was reduced in mixed infections, as the majority of these fish cleared the infection by the end of the study. Controlled laboratory studies with single and mixed treatments of genotypes I and II may identify how these different genotypes interact within the salmon host and the mechanism regulating the interaction. An understanding of this could improve prediction of infection outcomes on a population level by coupling epidemiological models to a within-host model incorporating mixed infections (Mideo et al. 2008); Chinook salmon and *C. shasta* would provide an appropriate aquatic system in which to develop this approach.

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CHAPTER 3: LINKING WITHIN-HOST COMPETITION TO PARASITE DYNAMICS IN A RIVER SYSTEM

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ABSTRACT

We investigated within-host parasite competition in Chinook salmon using two genotypes of the myxozoan *Ceratonova shasta*, which provides a unique system as it has both micro- and macroparasite traits. We hypothesized that within-host competition would occur between the genotypes when encountered concurrently, with the more virulent genotype as the better competitor. In contrast, sequential exposure would provide an advantage for the first infecting genotype. A laboratory study was conducted with Chinook salmon exposed to each genotype alone, to both genotypes concurrently and to both genotypes sequentially. We then assessed parasite virulence (host mortality), replication rate and mature parasite production across all treatments. As hypothesized, concurrent mixed parasite genotype infections resulted in a reduction in the number of mature parasites (competition) compared to single genotype infections. This reduction occurred only for the less virulent genotype, providing evidence that the more virulent genotype was a better competitor. When fish were exposed to each parasite genotype sequentially, the first infecting genotype produced the majority of mature parasites, suggesting an advantage for the prior resident. We discuss the potential role of within-host parasite interactions in determining parasite genotype dominance in a river system and in relation to theories about parasite genotype virulence evolution. We also suggest parasite genotypes have a facultative response to a competitor.

INTRODUCTION

Mixed infections, with more than one parasite or parasite strain infecting a single host, are common (Read and Taylor 2001; Pederson and Fenton 2007; Telfer et al. 2010) and the interactions between parasites can alter infection/disease dynamics (Cox 2001; Gower and Webster 2005; Bell et al. 2006; Mideo 2009; Telfer et al. 2010). Parasite interactions can be either facilitative or competitive, although competitive interactions seem to occur more often, especially in studies that examine interactions between strains of the same parasite species. For example, studies of *Plasmodium chabaudi* Landau, *Trypanosoma brucei* Plimmer & Bradford and *Schistosoma mansoni* Sambon all found that interactions between parasite strains were competitive, as evidenced by reductions in within-host replication and mature parasite production (de Roode et al. 2005a, b; Gower and Webster 2005; Balmer et al. 2009). This is likely because strains of the same parasite often have overlapping niches and elicit similar immune responses (Mideo 2009).

Within-host competition theory suggests that more virulent parasite strains or species will be better competitors due to tradeoffs in life history traits associated with high virulence, such as higher within-host growth rates (May and Nowak 1995; van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998). This was supported in mixed strain infections of *P. chaubaudi* and *T. brucei*, where the more virulent parasite strain was the better competitor (de Roode et al. 2005a, b; Balmer et al. 2009). The exception was snails (*Biomphalaria glabrata* Say) infected with *S. mansoni,* in which the less virulent strain was the better competitor (Gower and Webster 2005). In this case the less virulent strain maintained a higher growth rate, which likely accounted for its competitive superiority.

When exposures to different parasite strains occur sequentially, a parasite that is competitively inferior in a concurrent infection may gain a competitive advantage as a result of prior residency (Lello 2012). In mice infected with *P. chaubaudi*, parasite replication was unchanged for the first infecting strain, but decreased for the second strain, suggesting a competitive advantage for the initial strain (de Roode et al. 2005b). In contrast, the less virulent strain, whether it was introduced first or second, competitively suppressed the more virulent strain in snails exposed

sequentially to *S. mansoni* (Gower and Webster 2005). Because systems investigating sequential exposures to two strains of the same parasite are limited, we expand our comparison to include a bacterial (*Pasteuria ramosa* Metchnikoff and its host *Daphnia magna* Straus; Ben-Ami et al. 2008) and viral system (two serotypes of *Dengue virus* in a mosquito; Pepin et al. 2008). In both systems, the first infecting bacterial strain or viral serotype produced more spores or virus respectively, indicating that a competitive advantage for the initial strain is common.

Here, we examine within-host competition between two genotypes of the myxozoan parasite, *Ceratonova shasta* (Noble) in its Chinook salmon (*Oncorhynchus tshawytscha* Walbaum) host. This parasite is unique compared to the other systems mentioned above because it has traits of both micro and macroparasites. In addition, Chinook salmon are an important natural resource and recent declines in returning adults in at least one river have been linked, in part, to *C. shasta* infections (Fujiwara et al. 2011). Because Chinook salmon have a high prevalence of mixed genotype infections (Hurst et al. 2014), there is the potential that within-host interactions may be influencing parasite dynamics in the river system. Thus, we hypothesize that genotypes with different virulence levels compete within the host when infections are concurrent. We predict that the more virulent genotype will be the better competitor, suppressing the less virulent genotype. Lastly, we hypothesize that when fish encountered the genotypes sequentially, the first infecting parasite genotype would have a competitive advantage. By testing these hypotheses, we will gain a better understanding of how within-host parasite interactions affect infection outcomes. We will then use this information to help explain disease patterns in a natural system where the parasite is a serious pathogen and to predict pathogen evolutionary trajectories.

MATERIALS AND METHODS

Host-parasite system

To test our hypotheses of parasite genotype competition within the salmon host, two *C. shasta* genotypes were cultured in the laboratory. The salmon host becomes infected after the waterborne actinospore stage attaches to gill epithelia and invades

the gill blood vessels. The parasite then proliferates in the blood vessels and migrates to the intestine, where replication continues, culminating in maturation of the myxospore (Bjork and Bartholomew 2010). This stage is released into the water column and infects the polychaete host, *Manayunkia speciosa* Leidy, completing the life cycle (Fig. 3.1) with the production and release of the actinospore stage (Bartholomew et al. 1997).

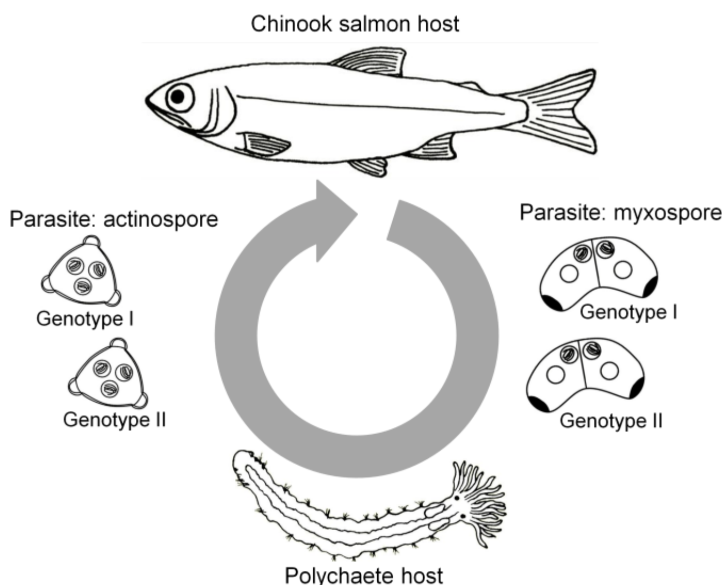

Figure 3.1 Life cycle of *Ceratonova shasta*.

Four parasite genotypes co-occur in river basins throughout the parasite's range in the Pacific northwest of North America, with genotypes I and II appearing to be the most common (Atkinson and Bartholomew 2010a, b; Hallett et al. 2012; Stinson 2012). Parasite-associated host mortality has been demonstrated with genotype I in Chinook salmon (Hurst and Bartholomew 2012). In contrast, genotype II infections in Chinook salmon are usually non-lethal (Atkinson and Bartholomew 2010b; Hurst and Bartholomew 2012). Life cycles for genotypes I and II have recently been established in the laboratory making investigation into within-host interactions possible in an allopatric strain of Chinook salmon (Salmon River Hatchery, Otis, OR, USA) that becomes infected and diseased at low parasite doses.

Establishment of infection

All animal challenges, husbandry and sampling were conducted at the John L. Fryer Salmon Disease Laboratory at Oregon State University, Corvallis, Oregon. Fish were exposed either singly (I or II), concurrently (I and II, I and I, II and II), or sequentially (I-I, II-II, I-II, II-I) to parasite genotypes I and/or II (summarized in Table 3.1). Treatment groups consisted of 90 juvenile Chinook salmon (~10g), with 30 salmon in each of the three replicates for a total of 1100 fish. Limited fish numbers required exceptions: the negative control consisted of two replicates of 25 salmon and single concurrent treatments had three replicates of 25 salmon. Each treatment consisted of two 24 h static exposures with the first on day 0 and the second on day 6. Specific-pathogen free well water was used as a mock exposure on day 6 for single and concurrent treatments (Table 3.1). Single parasite genotype exposures were conducted on day 0 and single parasite genotype delayed exposures on day 6 to control for potential differences in parasite viability between exposure days. Concurrent single genotype exposures (I and I, II and II) were conducted to control for total parasite dose (a target of 10 parasites fish^{-1}) in the mixed concurrent treatment (a target of 5 parasites fish^{-1} of each genotype, 10 total). Exposures to the same genotype sequentially were used as controls for mixed sequential treatments.

Table 3.1 Summary of parasite treatments and actual dose as measured by quantitative PCR. Juvenile Chinook salmon were exposed to genotype I, genotype II, both or specific-pathogen free well water. Genotype exposures were conducted either concurrently on day 0 or sequentially on days 0 and 6.

Treatment	Exposure		Parasites per Fish		Percent Mortality	Mean Day to Death
	Day 0	Day 6	Day 0	Day 6		
Control	Water	Water	0.0	0.0	0	N/A
Single I	I	Water	2.4 ± 0.8	0.0	100 ± 0	23 ± 0
Single delayed I	Water	I	0.0	2.6 ± 0.4	100 ± 0	23 ± 0
Single II	II	Water	3.0 ± 0.3	0.0	59 ± 3	40 ± 1
Single delayed II	Water	II	0.0	5.9 ± 1.4	56 ± 6	33 ± 1
Single concurrent I	I	Water	4.4 ± 0.7	0.0	100 ± 0	21 ± 0
Single concurrent II	II	Water	2.7 ± 0.3	0.0	40 ± 15	31 ± 2

Mixed concurrent	I and II	Water	5.9 ± 0.3 2.3 II, 3.6 I	0.0	100 ± 0	22 ± 0
Single sequential I	I	I	7.3 ± 0.3	3.1 ± 0.6	100 ± 0	23 ± 0
Single sequential II	II	II	2.1 ± 0.7	5.6 ± 0.6	39 ± 3	34 ± 2
Mixed sequential I-II	I	II	4.7 ± 0.7	4.2 ± 1.0	98 ± 2	24 ± 1
Mixed sequential II-I	II	I	1.6 ± 0.4	3.9 ± 1.4	100 ± 0	30± 0

To determine parasite dose, 3x1 L samples of water were collected from polychaete cultures producing either genotype I or II and filtered through a 5µm nitrocellulose membrane. Parasite density was assessed in the filtrates of each water sample using a *C. shasta*-specific qPCR assay (Hallett and Bartholomew 2006). Parasite dose was administered by adding 23 L of water containing a target of either ~5 or 10 parasites fish^{-1}, depending on treatment, at 18°C. An equal exposure dose was assumed to occur for all fish within a replicate tank. The experimental dose was validated by collecting 1 L of water from each treatment replicate and quantifying parasite density. Following exposure, flow of specific-pathogen free well water at 18°C was resumed. Salmon were fed a commercial fish diet daily (Bio-Oregon, Longview, WA, USA) and monitored for clinical disease signs.

Interaction measures

Five salmon from each treatment replicate were sampled on days 1, 7 and 14. Fish were euthanized using an overdose of tricaine methanesulfonate (MS-222, Argent Chemical Laboratories, Redmond, WA, USA), and the left gill (days 1, 7 and 14) and 0.25 mg of the posterior intestine (days 7 and 14) were collected and stored individually at -20°C until processing. Intestinal samples were not collected on day 1 because parasites are not present in intestinal tissue this early in the infection (Bjork and Bartholomew 2010). To assess parasite DNA quantity (parasite replication) in the gills and intestines, parasite DNA from each sample was extracted, purified (Hurst et al. 2014) and assayed using qPCR (Hallett and Bartholomew 2006). Inhibition tests were conducted on 10 randomly selected samples of each sample type (Hallett and Bartholomew 2009). A standard curve was created for estimating the parasite DNA

copy number in 0.1 g of host intestinal tissue by assaying ten-fold serial dilutions of a synthetic parasite template (Hallett and Bartholomew 2006) combined with intestinal tissue. A standard curve for either gill (y = -3.35x + 38.40; r^2 = 0.997; Hurst et al. 2014) or intestinal (y = -3.54x + 40.08; r^2 = 0.999) tissue was then used to calculate parasite copy number in the sample. To determine genotype proportion, DNA was sequenced and analyzed as in Hurst et al. (2014).

Fish that displayed terminal disease signs were euthanized; the date was recorded and used as a measure of genotype virulence. Intestines were excised, weighed, and 1 mL of tap water was used to flush mature parasites (myxospores) from the tissue into a microcentrifuge tube. Myxospores were counted using a hemocytometer and a 20 µL aliquot of this solution was retained for genotyping. For the single genotype treatments, harvested myxospores from 10 randomly selected fish were assayed to confirm genotype. For the mixed genotype treatments, genotype proportion was determined for all moribund fish.

Data Analyses

Survival among replicates within a treatment and among treatments was evaluated using a Mantel-Cox test. Replication in both the gill and the intestine was evaluated among replicates within a treatment and among treatments using a two-way ANOVA with Tukey's test for highly significant differences. Myxospore counts were transformed to the natural log scale for normality and differences among replicates within a treatment and among treatments were assessed using a one-way ANOVA with Tukey's test for highly significant differences. Comparisons of single and single-delayed genotype treatments were conducted using a student's t-test to assess the effects of parasite genotype viability on the different exposure days. To obtain genotype-specific myxospore counts, the proportion of each genotype from the sequencing assay was multipled by the total number of myxospores for each fish. These values were then natural log transformed and a one-way ANOVA with Tukey's test for highly significant differences was used to assess differences among replicates within a treatment and among treatments. For assessment of replication and myxospore counts, if no differences were detected among replicates, the mean for each replicate was calculated and the three values were used for comparisons among

treatments. All statistical analyses were performed using R and differences were considered significant at $P < 0.05$.

RESULTS

Parasite dose

The actual parasite dose per fish across all treatments was a mean of 3.7 parasites fish^{-1} (range 1.6-7.3) for the exposure on day 0 and a mean of 4.2 parasites fish^{-1} (range 2.6 to 5.9) for the exposure on day 6 (Table 3.1). Sequencing of parasite DNA from water samples confirmed that both single concurrent treatments contained only parasites of their respective genotype.

Virulence/host mortality

Fish infected with genotype I had a higher cumulative mortality and faster mortality rate than those infected with genotype II, confirming genotype I is more virulent (Fig. 3.2a; Mantel-Cox$_1$ = 109, $P < 0.001$). The cumulative mortality and mortality rate for genotype I was 100% in 23 days compared with 56 to 59% in 33-40 days for fish infected with genotype II. Cumulative mortality and mortality rate from single genotype exposures (day 0) and delayed single genotype exposures (day 6) did not differ significantly and therefore only the single genotype treatments conducted on day 0 were included in further analyses (Mantel-Cox$_1$ = 0.45, $P = 0.503$ for genotype I; Mantel-Cox$_1$ = 0.45, $P = 0.547$ for genotype II).

The cumulative mortality of salmon (Fig. 3.2a) with mixed concurrent infections (I + II) was 100%, the same as fish exposed only to genotype I (Table 3.1). Although the mortality rate for the mixed concurrent treatment was significantly faster (mean of 22 days) than the single I treatment (mean of 23 days; Mantel-Cox$_1$ = 18.8, $P < 0.001$), it was slower than the single concurrent I (I + I) treatment (mean of 21 days; Mantel-Cox$_1$ = 18.0, $P < 0.001$), demonstrating that a higher dose of genotype I results in a faster mortality rate.

Among fish exposed to parasite genotypes sequentially (Fig. 3.2b; Mantel-Cox$_3$ = 203.9, $P < 0.001$), the highest cumulative mortality and fastest mortality rate

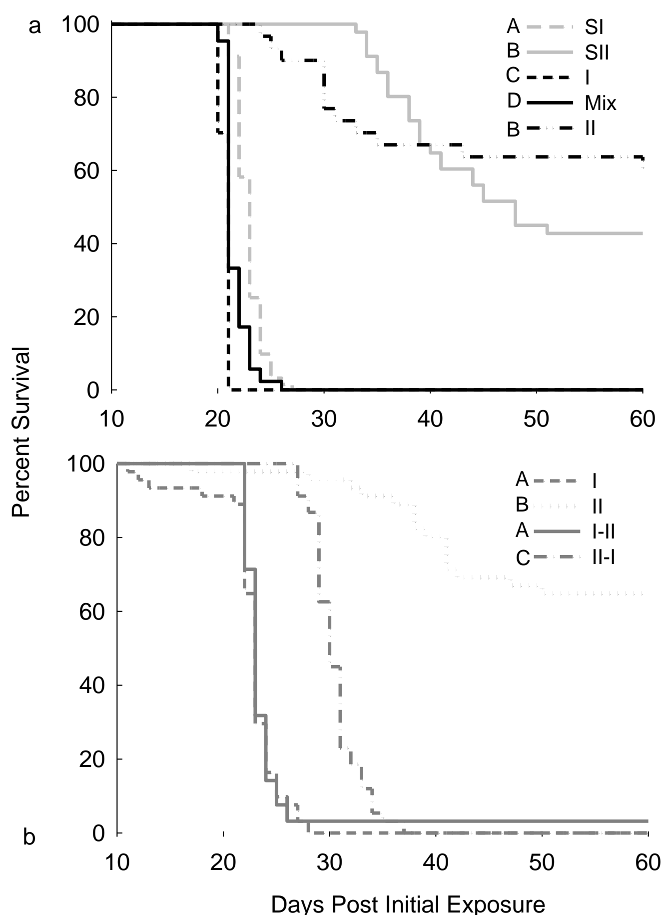

Figure 3.2 Percent survival for a) single (SI or SII) and concurrent treatments and b) sequential treatments. I or II = genotype I or II. Letters indicate significant differences among treatments as determined by a Cox proportional hazards test.

occurred in fish exposed to genotype I only (100%, mean of 23 days) and the mixed sequential treatment with initial exposure to genotype I (98%, mean of 24 days). These treatments differed significantly from the mixed sequential treatment with exposure to genotype II first (100%, mean of 30 days). However, the slower mortality rate can be attributed to the delayed exposure to genotype I. The lowest mortality and slowest mortality rate occurred in fish exposed sequentially to genotype II only (39%, mean of 34 days), demonstrating that virulence is affected by genotype but not by order of infection.

Parasite replication

For all single and concurrent treatments (Figs 3.3a and 3.3b), a two-way ANOVA showed that total parasite copy number increased over time ($F_{2,30}$ = 218.14, P < 0.001 gill; $F_{1,20}$ = 753.089, P < 0.001 intestine) and differed among treatments ($F_{4,30}$ = 49.53, P < 0.001 gill; $F_{4,20}$ = 24.447, P < 0.001 intestine). The interaction of treatment and day in the gill ($F_{8,30}$ = 6.6, P < 0.001), but not the intestine ($F_{4,20}$ = 0.585, P = 0.677) indicates that differences are difficult to detect in the early stages of infection in certain tissues (e.g. day 7 in the intestine). Examination of parasite genotype I replication in the gills using a one-way ANOVA with only treatment as a factor, showed that parasite replication in the single concurrent I treatment was higher than in either the mixed or single I treatments ($F_{2,6}$ = 10.3, P =0.0114), likely due to the increased exposure dose of parasite genotype I. In the intestine on day 14, copy number was again higher in the concurrent compared with single treatments and could be attributed to the higher parasite exposure doses in the concurrent treatments.

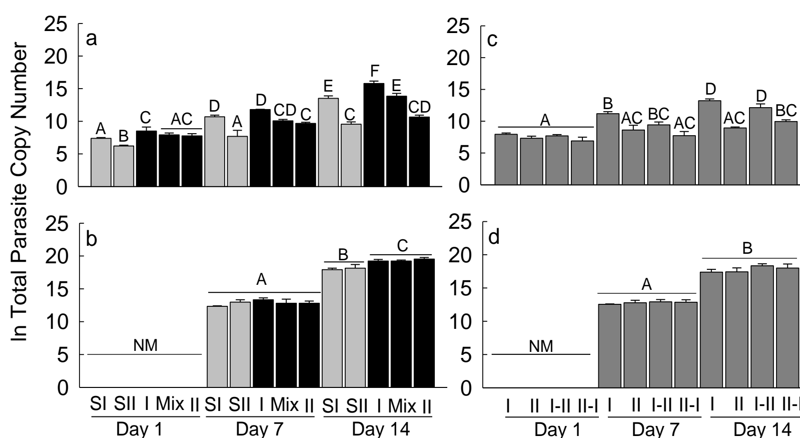

Figure 3.3 Mean *Ceratonova shasta* copy number (replication) in gill tissue sampled on days 1, 7 and 14 in a) single (SI or SII) and concurrent treatments and b) sequential treatments. Mean replication in intestinal tissue sampled on 7 and 14 days post exposure is shown in c) for single and concurrent treatments and d) for sequential treatments. NM= not measured. Letters indicate significant differences among treatments of each tissue using a two-way ANOVA with Tukey's test for highly significant differences.

Sequential exposures resulted in similar patterns of total parasite replication in the gills (Fig. 3.3c) as concurrent exposures; parasites increased over time among all treatments (treatment, $F_{3,24}$ = 23.617, P < 0.001; day $F_{2,24}$ = 66.254, P < 0.001; treatment:day, $F_{6,24}$ = 4.015, P = 0.006). Total parasite copy number in the intestine (Fig. 3.3d) also increased over time ($F_{1,16}$ = 294.349, P < 0.001), but did not differ

among treatments ($F_{3,16} = 1.074$, $P = 0.388$). Fish exposed to parasite genotype I first had higher replication rates in the gills than those initially exposed to parasite genotype II. However, when genotype II was administered first, replication of genotype I was reduced in both the gill ($F_{2,6} = 15.2$, $P = 0.005$) and intestine ($F_{2,6} = 7.16$, $P = 0.0257$; data not shown). This slower rate of replication can likely be attributed to the delay in the exposure to parasite genotype I.

Mature parasite production

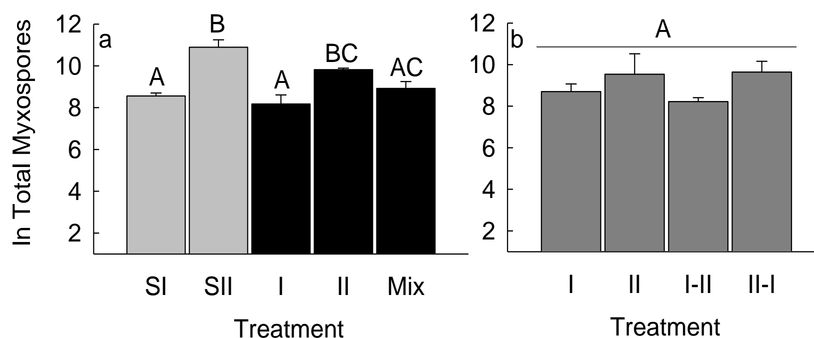

Figure 3.4 Mean myxospore counts of *Ceratonova shasta* for a) single (SI or SII) and concurrent treatments and b) sequential treatments. Letters indicate significant differences using a one-way ANOVA followed by a Tukey test for highly significant differences.

A one-way ANOVA revealed that the total quantity of myxospores differed among single and concurrent treatments (Fig. 3.4a; $F_{4, 10} = 15.8$, $P < 0.001$). The single delayed genotype treatments were not significantly different from the single treatments ($t_5 = 1.66$, $P = 0.212$ genotype I; $t_5 = 0.488$, $P = 0.666$ genotype II) ensuring parasite genotype viability was similar among exposure days. Thus, the single-delayed treatments were not included in further analyses. Pair-wise comparisons demonstrated that fewer myxospores were produced in fish infected with either parasite genotype I or both genotypes concurrently than fish infected with genotype II. The reduction in total myxospores in the mixed concurrent treatment was attributable to a decrease in genotype II (Figs 3.5a and 3.5b; $F_{2,6} = 19.2$, $P = 0.00247$).

Neither total myxospore production (Fig. 3.4b; $F_{3,8} = 0.973$, $P = 0.452$) nor genotype I-specific myxospore production (Fig. 3.5c; $F_{2,6} = 1.83$, $P = 0.24$) differed significantly among sequential treatments. Analysis of genotype-specific myxospore production demonstrated that genotype II myxospores were significantly reduced when fish were exposed to genotype I prior to genotype II (Fig. 3.5d; $F_{2,6} = 12.6$, $P = 0.0071$).

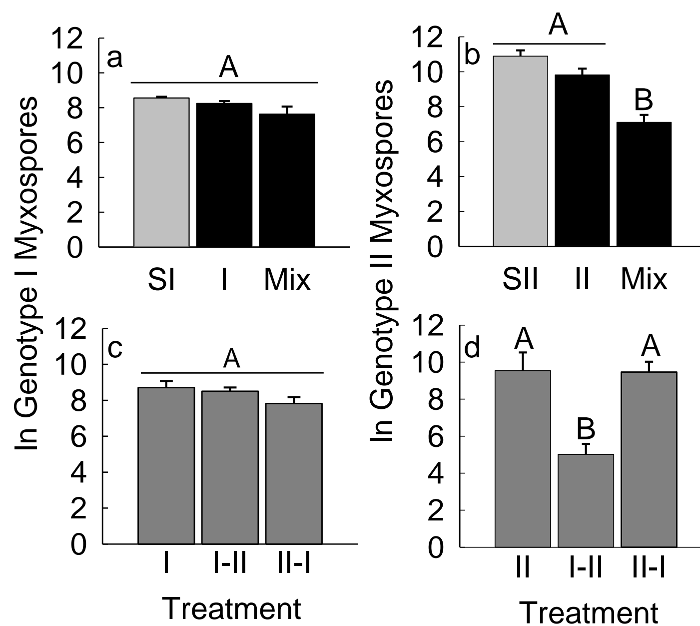

Figure 3.5 Mean *Ceratonova shasta* genotype I (a, c) and genotype II (b, d) myxospore counts from single (SI or SII), concurrent and sequential treatments. Letters indicate significant differences among treatments using a one-way ANOVA followed by Tukey's test for highly significant differences.

DISCUSSION

Does within-host competition occur?

Investigation of within-host interactions is essential for fully understanding the dynamics of a parasite in any given system. In our experimental system using two genotypes of *Ceratonova shasta* that infect Chinook salmon, there was evidence for competitive interactions between the two genotypes. Whether exposure to both genotypes occurred concurrently or the initial exposure was to genotype I, the number of genotype II myxospores decreased compared to infection with genotype II alone (Table 3.2). Although not the first study to demonstrate within-host genotype

competition using a parasite (de Roode et al. 2005a, b; Gower and Webster 2005; Balmer et al. 2009), ours is the first to do so in a vertebrate host from an outbred population, improving our ability to extrapolate the results of a laboratory study to a natural system.

Table 3.2 Summary of study findings

Exposure	Host Mortality	Host Mortality Rate	Replication Rate	Total Myxospore Production	Genotype Myxospore Production
Single I	High	Fast	Fast	Low	Low
Single II	Low	Slow	Slow	High	High
Mix I and II	High	Fast	Fast	Low	Low I, Low II
Mix I then II (I-II)	High	Fast	Fast	Low	Low I, Low II
Mix II then I (II-I)	High	Fast	Slow	High	High II, Low I

Competition can occur through a variety of mechanisms including direct interference, the host immune system and limited resources (Read and Taylor 2001). The limitation of host resources for genotype II due to the high virulence in mixed infections is one explanation for the competitive suppression of genotype II. A limited resource (e.g. available host red blood cells) has also been hypothesized as one mechanism regulating within-host competition in the malarial system (Bell et al. 2006).

The costs and benefits of being a better competitor

Similar to other parasite systems (de Roode et al. 2005b; Ben-Ami et al. 2008), competition was asymmetric in that only genotype II was suppressed, providing support for our hypothesis that the more virulent genotype (I) is the better competitor. Typically, a better competitor has a faster growth rate, which often correlates with selection for higher virulence as the parasite uses host resources more quickly. However, a more rapid death limits the time available for the production of transmission stages (May and Nowak 1995; van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998). Genotype I corroborated the theoretical predictions

of a better competitor by having a faster replication rate and higher virulence, but also in producing fewer myxospores than genotype II. Therefore, when Chinook salmon are infected by both genotypes, genotype I will have higher fitness because it can replicate faster and the host succumbs to infection before genotype II can reach its full transmission potential. During single infections, the lower virulence of genotype II allows for increased myxospore production time and ultimately higher fitness.

Prior residence can eliminate competitive suppression

The ability of genotype II to overcome competitive suppression with prior residency may be a result of a facultative parasite response (conditional alteration of parasite behavior; Gower and Webster 2005) to a more virulent competitor. When genotype II was afforded residency prior to genotype I, fish produced similar numbers of genotype II myxospores as fish exposed only to genotype II. However, mortality occurred earlier and was higher than for single genotype II infections. One explanation is that the presence of genotype I induced a facultative response by genotype II to produce the same number of myxospores more rapidly. A facultative response has also been hypothesized for sequential infections of *S. mansoni* strains, whereby the low virulence strain increases its replication in response to a high virulence strain (Gower and Webster 2005). Parasite genotype recognition within the host is akin to chemical signaling (quorum sensing) in bacteria (Waters and Bassler 2005) and could be an evolutionary adaptation to competition in mixed infections (Read and Taylor 2001).

Within-host competition influences the infection outcome and genotype dominance

Competition between parasite genotypes can lead to three interaction outcomes: coinfection, where both genotypes coexist within the host, superinfection, where one genotype excludes the other and single infection, where a host becomes immune to infection by other genotypes (Nowak and May 1994; May and Nowak 1995; Mosquera and Adler 1998). The production of myxospores by both parasite genotypes during both sequential and concurrent mixed infections demonstrates that coinfection occurred in our experimental system. Mosquera and Adler (1998) demonstrated theoretically that coinfection favors higher virulence evolution of both

genotypes of a single parasite species. Therefore, absent other selective pressures, increased virulence seems to be the most likely evolutionary trajectory for both *C. shasta* parasite genotypes.

When extrapolating to a natural system, our experimental results are confounded by the heterogeneity in susceptibility to *C. shasta* among hosts in a natural population. In the malarial experimental system, host susceptibility was associated with infection outcome in that coinfection occurred in susceptible hosts and superinfection occurred in resistant hosts (de Roode et al. 2004). Parasite genotype interactions within the susceptible strain of Chinook salmon used in our study are likely more indicative of the dynamics taking place in adult salmon. As a species that dies after spawning, these fish are immunocompromised (Robertson et al. 1961) and susceptible to infection upon their return as adults to spawn in freshwater. Thus, the ability of both parasite genotypes to successfully produce mature parasite stages through coinfection may be limited to the more susceptible adult life stage.

Juvenile Chinook salmon from the Klamath River, OR-CA, are inherently more resistant to *C. shasta* than the Chinook strain used in our experiment as a result of their sympatric residence with the parasite (Bartholomew 1998). Despite this resistance, over 80% of naturally exposed juvenile Chinook salmon examined had mixed infections with parasite genotypes I and II (Hurst et al. 2014). In contrast with our laboratory results, where concurrent exposures resulting in disease led to coinfection, resistant juvenile Chinook salmon that became diseased produced only genotype I myxospores (Atkinson and Bartholomew 2010a, b; Hallett et al. 2012; Hurst et al. 2014). Therefore, superinfection, which excludes parasite genotype II, is the most likely infection outcome in resistant juvenile Chinook.

The order of infection coupled with the susceptibility of the salmonid host life stage present likely influences what parasite genotypes dominate in a river system. In the Klamath River, genotype I is dominant, but both genotypes are present where fish would encounter the parasite (Hallett et al. 2012), suggesting that concurrent mixed infections are more likely than sequential infections. Thus, both adults and juveniles maintain parasite genotype I dominance, through coinfection and superinfection respectively. However, coinfection in the adults may also account for the continued

persistence of genotype II in years where the returns of salmonid hosts associated with genotype II, such as coho salmon (*O. kisutch* Walbaum), are low.

Implications for salmonid reintroductions and barrier removal

With managers striving to restore river systems to a more natural state considering the removal of anthropogenic barriers and reintroducing salmonids, knowledge of within-host interactions could be helpful for predicting how pathogens may be redistributed and in selecting salmonid stocks with the best chance for survival. For example, due to anthropogenic barriers in the Klamath River Basin OR-CA, Chinook salmon are prevented from migrating into their historic habitats in the upper part of the basin and their exclusion in these reaches is associated with the absence of genotype I in the upper basin (Atkinson and Bartholomew 2010a; Hurst et al. 2012). The removal of these barriers could result in early/prior exposure of Chinook salmon to genotype II. In the Klamath system, the most likely stock used for reintroduction is the resistant juvenile Chinook that currently reside there. Over time, genotype I would be introduced above the barriers with migrating adult salmon and the system would remain dominated by genotype I due to superinfection. However, if a susceptible Chinook salmon stock was chosen, our study suggests that genotype II would have an advantage. By allowing sequential infections to become more common, genotype II may increase in the system due to its ability to eliminate competitive suppression.

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CHAPTER 4: WHAT CAN CYTOKINE EXPRESSION REVEAL ABOUT THE ECOLOGY OF PARASITE GENOTYPE INTERACTIONS?

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ABSTRACT

The parasite *Ceratonova shasta* is comprised of multiple genotypes with differing fitness in the Chinook salmon (*Oncorhynchus tshawytscha)* host. These differences led to the hypothesis that the host immune response to single genotype infections varies depending on the parasite genotype. When two genotypes coinfect the host, we predicted that the host immune response to each genotype is elicited simultaneously and assists in mediating competitive interactions between parasite genotypes. The host immune response to each parasite genotype during single infections was unique, as indicated through analysis of the systemic (spleen) and local (intestine) cytokine profiles at days 1, 7 and 14 post infection. For the more virulent parasite genotype I, the host response was delayed, primarily systemic, and the upregulation of inflammatory cytokines (IFNγ, IL-6) was eclipsed by a large regulatory cytokine response (IL-10) leading to immunosuppression. In contrast, the host response to the less virulent parasite genotype II was most evident locally by the upregulation of inflammatory cytokines (IFNγ, IL-6). During coinfection, parasite-genotype specific responses were elicited simultaneously; the systemic response to coinfection was similar to the parasite genotype I response, whereas the local response was more similar to the parasite genotype II response. A partially effective host response to parasite genotype II explains the decrease in genotype II during competitive interactions, but the high host mortality is likely due to the systemic immunosuppression associated with parasite genotype I.

INTRODUCTION

Coinfection with multiple pathogen strains is common (Telfer et al. 2010) and can sometimes lead to pathogen competition (measured as a decrease in one or both infecting pathogens' abundance). This within-host competition can alter pathogen strain growth and persistence within the host (Read and Taylor 2001) and influence virulence evolution (May and Nowak 1995; Frank 1996; Mosquera and Adler 1998). However, the direction of virulence evolution depends on the mechanism(s) by which competitive interactions (i.e. direct interference, resource limitation and immune mediation) are regulated (Read and Taylor 2001; Råberg et al. 2006; Mideo 2009). Although the evolution of virulence can be influenced by a variety of selective pressures, we focused on the potential for immune mediation in this study and detail a few possible outcomes. If the immune system is non-specific, then an increased immune response to one pathogen strain confers host protection to related strains (Gupta et al. 1998; Read and Taylor 2001) leading to selection for higher virulence. If pathogen strain-specific immunity targets one strain preferentially, then the other strain may "escape" the immune response, leading to selection for reduced virulence (Råberg et al. 2006). Thus, the immune system can indirectly mediate competition between pathogen strains and over time can alter strain virulence.

We have previously identified a competitive interaction between two genotypes of the myxozoan parasite *Ceratonova shasta* during coinfection in Chinook salmon (*Oncorhynchus tshawytscha*; Hurst and Bartholomew submitted). Similar to other myxozoans, *C. shasta* has a complex life cycle that requires both a definitive invertebrate host and an intermediate vertebrate host, with distinct spores formed in each. The invertebrate host, *Manayunkia speciosa*, is a freshwater polychaete that becomes infected by the waterborne myxospore released from the vertebrate host. The parasite replicates within the polychaete and then mature actinospores are released into the water column (Bartholomew et al. 1997). The actinospore infects a variety of salmon and trout species, with initial attachment to the gill followed by proliferation in the blood and migration to the intestine where the parasite eventually matures into the myxospore (Bjork and Bartholomew 2010). Release of mature

myxospores typically occurs in salmon at death and is associated with intestinal hemorrhaging and necrosis and a swollen vent (Bartholomew et al. 1998).

Variations in the fitness and virulence (harm to the host) of *C. shasta* genotypes I and II in single and coinfections in Chinook salmon (Hurst and Bartholomew 2012; Hurst and Bartholomew submitted) suggested the host immune response may have regulated the outcome of the various infection scenarios. The slower replication, lower virulence and greater myxospore production of parasite genotype II compared to genotype I during single infections in the same host strain (Hurst and Bartholomew submitted) led to the hypothesis that the immune response is parasite genotype-specific. Analyses of cytokine expression supported our hypothesis that single infections elicited a host response that was parasite genotype-specific. Previously, Hurst and Bartholomew (submitted) found that coinfection led to competitive interactions between parasite genotypes that resulted in fast replication and high virulence similar to single infections with genotype I, suggesting genotype I outcompeted genotype II and gained an advantage. Thus, we hypothesized that during coinfection, dual and potentially conflicting immune responses would render the overall immune response ineffective. Our data suggested that the specific-responses to each genotype were elicited simultaneously, with a partially effective immune response to genotype II explaining the reduction in genotype II, and the immunosuppressive response to genotype I accounting for the fast replication and high virulence measured during coinfection.

MATERIALS AND METHODS

Experimental Design

The experimental design was detailed previously (Hurst and Bartholomew submitted) and is briefly summarized here. Samples for measuring cytokine expression in this study were collected from juvenile Chinook salmon infected with parasite genotype I, genotype II, genotypes I and II and an uninfected control for a total of 330 samples. Fish were exposed to 2.4 ± 0.8 (genotype I), 3.0 ± 0.3 (genotype II) and 5.9 ± 0.3 (3.6 genotype I, 2.3 genotype II) parasites fish^{-1}. After euthanizing with an overdose of tricaine methanesulfonate, five fish from each of three treatment

replicates were sampled at 1, 7 and 14 days post exposure. A 25 mg portion of the intestine (the parasite's target tissue, used to measure the local response) and spleen (used to measure the systemic response) were collected, preserved in RNALater (Qiagen) and frozen at -80 °C. RNA was extracted from tissues using the Roche High Resolution RNA Tissue Kit and was then converted to cDNA using the Roche Transcriptor First Strand cDNA Synthesis Kit with oligo DT primers.

Gene expression

Cytokines assayed included: three inflammatory cytokines (TNFα, IL-1β, IFNγ), two regulatory cytokines (TGFβ, IL-10), IL-6 (both inflammatory and regulatory) and β-actin as a reference (Table 4.1). Cytokine and primer selection was based on the results of the cytokine profile used in Bjork et al. (2014) for investigating the immune response of Chinook salmon to *C. shasta.* The authors demonstrated upregulation of IFNγ, IL-10 and IL-6 in response to infection. Although no significant upregulation of TNFα, IL-1β or TGFβ occurred, we included these cytokines in our study because they are typically expressed early in response to pathogen infection.

Table 4.1 Cytokines assayed and their function in teleosts.

Cytokine	Function
TNFα	Inflammatory; induction of macrophage responses to pathogens including phagocytosis, reactive oxygen and nitric oxide production (Grayfer et al. 2008)
IL-1β	Early inflammatory; stimulates itself along with other molecules including IL-6 (Huising et al. 2004; Yang et al. 2013).
IFNγ	Inflammatory; similar in innate function to TNFα, recruits cells to the infection site through the use of chemokines and promotes antigen presentation (Robertson 2006)
IL-6	Inflammatory and regulatory; involved in the acute phase response which stimulates inflammation, but also inhibits TNFα and IL-1β and activates IL-10 to control inflammation (Chen et al. 2012).
TGFβ	Regulatory; inhibits lymphocyte proliferation (Yang and Zhou 2008)
IL-10	Regulatory; downregulates expression of TNFα, IL-1β and IFNγ (Grayfer et al. 2011).

Cytokine expression was measured using qPCR according to Bjork et al. (2014). Two µL of cDNA from each of the five fish per treatment replicate were pooled and diluted 1:10 then assayed in duplicate. The log fold change in cytokine expression and standard error from each treatment was calculated and compared to uninfected controls using REST 2009 software (Pfaffl et al. 2002; Qiagen) with 2000 randomizations (Bjork et al. 2014). Treated samples were considered significantly different from controls at $p < 0.05$.

RESULTS

Systemic response

Cytokine profiles are often a good indicator of the type of immune response to a particular infection. Significant changes in systemic cytokine expression occurred, with higher expression in fish infected with parasite genotype I than genotype II (Fig. 4.1). In fish infected with parasite genotype I, there were no significant changes in expression compared to uninfected controls until day 14 when upregulation of IFNγ, IL-10 and IL-6 ($P < 0.001$) became apparent. The systemic response to parasite genotype II was more rapid, with significant downregulation of TNFα ($P = 0.033$) and IL-6 ($P = 0.033$) on day 1 and IL-1β on day 7 ($P = 0.033$). Significant upregulation of TGFβ occurred on day 1 ($P < 0.001$) and day 7 ($P < 0.001$). By day 14, IFNγ and IL-10 were both upregulated in response to parasite genotype II ($P < 0.001$), but were approximately 2.5 and 7 fold lower, respectively, than the host response to genotype I.

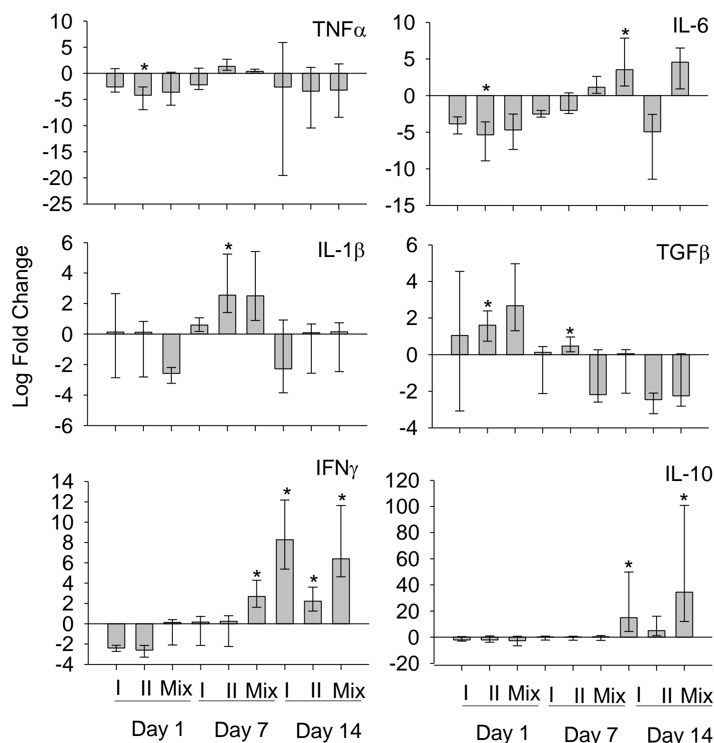

Figure 4.1 Expression of inflammatory and regulatory cytokines in spleen tissue. Log fold change values and standard error were calculated using REST Software (2009) and includes three replicate spleen samples using pools of extracted RNA from five fish in each replicate. An asterisk indicates values significantly different from the control. Fish were sampled on days 1, 7 and 14 from fish exposed to *Ceratonova shasta* genotype I (I), genotype II (II) or a mixture of both genotypes (mix).

The response to coinfection with parasite genotypes I and II was similar to the host response to genotype I with a few exceptions. By day 14, both IFNγ and IL-10 significantly increased ($P < 0.001$) compared to uninfected controls, as observed for parasite genotype I (Fig. 4.1). However, there was a more rapid response to coinfection, as evidenced by the significant upregulation of IFNγ at day 7. Interestingly, IL-10 expression at day 14 was more than double the expression of IL-10 in response to either parasite genotype alone.

Localized response

In single infections of either parasite genotype, IL-1β was significantly downregulated during all sampling times ($P < 0.001$), despite its role as an inflammatory cytokine (Fig. 4.2). By day 14, IL-6 was significantly upregulated for both parasite genotypes ($P < 0.001$) compared to uninfected controls, but was 5-fold higher for genotype II. Although IFNγ expression increased in fish infected with parasite genotype I, it was not significant. Interestingly, expression of IFNγ was

significantly upregulated for the less virulent parasite genotype II ($P < 0.001$) to approximately 3-fold higher than genotype I. Although IL-10 was downregulated on day 7 for both parasite genotypes ($P < 0.001$ genotype I; $P = 0.038$ genotype II), expression appears to increase by day 14. This coincided with a significant upregulation in TGFβ for parasite genotype I ($P < 0.001$), with a similar trend for parasite genotype II.

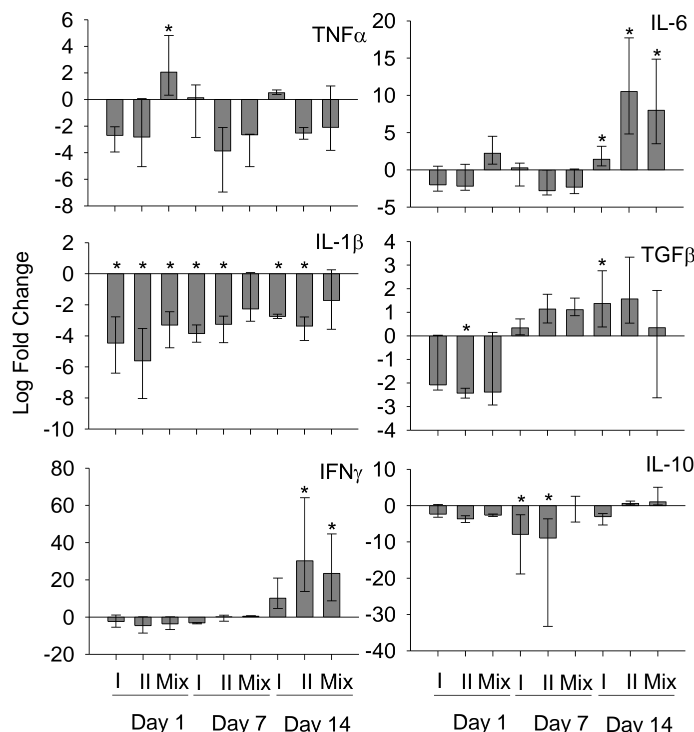

Figure 4.2 Expression of inflammatory and regulatory cytokines in intestinal tissue. Log fold change values and standard error were calculated using REST Software (2009) and includes three replicate intestinal samples using pools of extracted RNA from five fish in each replicate. An asterisk indicates values significantly different from the control. Fish were sampled on days 1, 7 and 14 from fish exposed to *Ceratonova shasta* genotype I (I), genotype II (II) or a mixture of both genotypes (mix).

The local response to coinfection was similar to the response to genotype II, with the exception of the significant upregulation of TNFα ($P < 0.001$) on day 1 (Fig. 4.2). By day 14, expression for both IFNγ ($P < 0.001$) and IL-6 ($P = 0.033$) increased significantly, akin to the fold change observed for genotype II. IL-1β expression was downregulated similarly among co- and single infections, although downregulation during coinfection was only significant on day 1 ($P < 0.001$), likely because of the variation among replicates. The expression of the regulatory cytokines TGFβ and IL-

10 did not change significantly in response to coinfection, indicating a minimal local regulatory response.

DISCUSSION

Infection of Chinook salmon with different genetic variants of *C. shasta* resulted in variations in both parasite fitness and virulence. We hypothesized that differences in the infection outcome with each parasite genotype could be explained by the elicitation of a unique host immune response. When fish were infected with a single parasite genotype, the location and timing of cytokine expression differed, suggesting that the host immune response is parasite genotype-specific. The delayed systemic response to parasite genotype I compared to genotype II may be a result of this parasite strain being able to initially evade the immune response, as has been suggested for other myxozoan parasites (Sitja-Bobadilla 2008). Expression of inflammatory cytokines (IFNγ, IL-6) against parasite genotype I was delayed and coincided with even higher expression of IL-10. Thus, the inflammatory response may have been regulated before it could effectively limit parasite replication/damage, leading to the faster replication and higher virulence of this parasite genotype in Chinook salmon (Hurst and Bartholomew submitted). Conversely, the high expression of inflammatory cytokines (IFNγ, IL-6) locally and the more rapid host response to parasite genotype II both locally and systemically, likely contributed to the effective control of disease progression in fish infected with only genotype II (Hurst and Bartholomew submitted). Thus, the host immune response to parasite genotype I was primarily systemic and immunosuppressive, while the response to parasite genotype II was more rapid and likely caused only localized inflammation.

In coinfected Chinook salmon, the competitive interaction resulting in a decrease in genotype II (Hurst and Bartholomew submitted) appears to be mediated indirectly through a host immune response comprised of elements from each parasite genotype-specific response. The systemic host response to coinfection was similar to genotype I, with upregulated expression of both inflammatory (IFNγ, IL-6) and regulatory (IL-10) cytokines. The local response to coinfection was similar to that targeting parasite genotype II, with a more rapid response time and an inflammatory

response facilitated by the upregulation of IFNγ and IL-6. Although the decrease in parasite genotype II may have been due to the elicitation of a partially effective genotype II-like local response, the fast replication and high virulence were similar to infections with parasite genotype I (Hurst and Bartholomew submitted). This suggests that the overall immune response was ineffective due to the systemic immunosuppression elicited by parasite genotype I and thus provided a greater advantage for genotype I during coinfection.

Despite the evidence for mediation of parasite genotype competitive interactions through immunosuppression, we cannot rule out additional mechanisms. Researchers working with the protozoan parasite *Plasmodium chabaudi* in murine models found evidence of both immune and resource related mechanisms mediating parasite strain interactions. Furthermore, the dominance of a certain mechanism likely changed over the course of the infection. For example, resources (the availability of red blood cells) or innate immunity regulated interactions early in the infection, while adaptive immunity (T-cell dependent) mediated interactions late in the infection (Råberg et al. 2006). Thus, for *C. shasta*, competition may be mediated by a combination of immunosuppression and either limited resources or direct interference between genotypes. Hurst and Bartholomew (submitted) suggested that limited host resources brought about by the high virulence in coinfection was partly responsible for the decrease in genotype II compared with fish that had single genotype II infections. Therefore, immunosuppression may have allowed genotype I to continue to replicate more quickly and achieve competitive superiority, while the shortened host life span and genotype II-specific immune response limited genotype II.

One of the virulence mechanisms many parasites possess is protease production and differences in production between genotypes may be one avenue through which genotypes can directly interfere with one another. Protease genes have been identified in myxozoan parasites including cathepsin-Z like and serine proteases in *Myxobolus cerebralis* (Kelley et al. 2003; Kelley et al. 2004) and a cathepsin-L like protease in *Kudoa paniformis* and *K. thyrsites* (Funk et al. 2008). These enzymes have many functions in pathogenesis including immune evasion, immune activation, immune suppression, degradation of blood proteins and involvement with parasite

invasion through tissue (McKerrow et al. 2006). For example, *Entamoeba histolytica* produces a cysteine protease that can inhibit the inflammatory cytokine IL-18 (McKerrow et al. 2006). In the *C. shasta* system, differences in the amount or type of proteases being produced could influence the outcome of both single and coinfections. For example, a larger amount of proteases produced by the more virulent genotype I may explain differences in virulence between infections with parasite genotype I and II. Perhaps proteases are produced in larger amounts by the more virulent genotype I. During coinfection, protease production may also mediate competitive interactions via direct harm to a competing parasite genotype, or indirectly, by limiting host resources (e.g. degrading host tissue).

The inflammatory cytokines IL-1β and TNFα are typically expressed early in response to pathogen infections. For the majority of treatments and sampling times included in our study, there was little change in expression in response to fish infected with *C. shasta* compared to controls. It is possible that our sampling times did not provide the resolution necessary to capture any significant upregulation. Researchers working with rainbow trout macrophage cell lines measured significant expression of both IL-1β and TNFα just five hours after incubation with an antigen (Zou et al. 2003). In another experiment, IL-1β expression was upregulated in rainbow trout infected with *M. cerebralis* as early as five minutes post exposure (Severin and El-Matbouli 2007). Thus, perhaps we missed the ideal window for measuring these two cytokines and earlier (12 hr post exposure) or more frequent sampling (additional sampling at 3 days and 5 days post exposure) may be needed to detect changes in their expression.

Even more interesting was the consistent downregulation of the inflammatory cytokine IL-1β in the intestine over the course of the study. Huising et al. (2004) demonstrated that teleost IL-1β is genetically distinct from IL-1β in other vertebrate species, indicating that its function may differ between teleosts and vertebrates. Experiments with rainbow trout infected with another myxozoan parasite, *Tetracapsuloides bryosalmonae*, demonstrated that the response to infection was dominated by lymphocytes, not macrophages, and IL-1β was not expressed, suggesting that eliciting a macrophage response coincides with upregulation of IL-1β

(Holland et al. 2003). A similar cellular response was observed in response to infection with *C. shasta* (Bjork et al. 2014). Therefore, it is likely that IL-1β is not expressed by Chinook salmon in response to infection with either *C. shasta* genotype pathway.

Conclusion

This study provides evidence for immune-mediation of the within-host competition between *C. shasta* genotypes I and II. The inflammatory response to the more virulent genotype I was delayed systemically and was eclipsed by a large regulatory response, indicative of immunosuppression. In contrast, the response to genotype II was more rapid, with expression of high levels of inflammatory cytokine (IFNγ, IL-6) expression occurring only locally. Coinfection elicited aspects of the response to each genotype for an overall immunosuppressive response. We suggest that the immune response was involved in the mediation of the competitive interaction between parasite genotypes, but may be working in conjunction with limited resources. A better understanding of the mechanisms regulating parasite interactions is important for predicting infection outcomes and the evolution of virulence within this parasite assemblage. Future studies could investigate if parasite proteases are involved with regulating competitive interactions as well as further characterize differences in the parasite genotype-specific immune response such as complement activity and the acute phase response in the liver.

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CHAPTER 5: IMMUNIZATION OF CHINOOK SALMON AGAINST *CERATONOVA SHASTA* USING A LESS VIRULENT PARASITE GENOTYPE

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ABSTRACT

The recent identification of multiple genotypes of the salmonid parasite *Ceratonova shasta* with different virulence levels in Chinook salmon (*Oncorhynchus tshawytscha)* suggests that it may be possible to immunize fish against subsequent infection and disease. We hypothesized that exposure of Chinook salmon to the less virulent parasite genotype (II) prior to the more virulent parasite genotype (I) would decrease disease and/or result in fewer mature parasites compared to fish only infected with the more virulent genotype. To test this, fish were challenged in a combination of field and laboratory exposures and we measured infection prevalence, percent morbidity, and mature parasite production. Neither mortality nor mature parasite production were reduced when fish were exposed to genotype II prior to genotype I as compared with fish exposed only to genotype I, suggesting that immunization with a less virulent parasite genotype does not occur.

INTRODUCTION

In aquaculture, there are opportunities for the control of diseases through vaccination and/or treatment. Vaccines have been developed for a variety of bacterial and viral pathogens, but no commercial parasite vaccines exist in aquaculture (Sommerset et al. 2005). However, there is evidence that fish can acquire resistance after a natural exposure to a parasite. In salmonids, resistance to parasite reinfection has been demonstrated for the microsporidians *Kabatana takedai* (Awakura 1974) and *Loma salmonae* (Speare et al. 1998; Kent et al. 1999), the monogenean *Discocotyle sagittata* (Rubio-Godoy and Tinsley 2004) and the myxozoan *Tetracapsuloides bryosalmonae* (Foott and Hedrick 1987). However, one drawback with using an immunization strategy based on exposure to a virulent parasite is the inability to achieve high infection prevalence without causing a high incidence of disease and/or mortality after the initial exposure. For example, Foott and Hedrick (1987) demonstrated that when infection prevalence with *T. bryosalmonae* was high (82%), most fish exhibited clinical disease signs.

Alternatively, immunization may also occur with prior exposure to attenuated parasites or naturally occurring less virulent parasite species/strains (Smith et al. 1999; Read and Taylor 2001). Attenuation decreases parasite virulence while simultaneously eliciting a protective immune response against future infections with an unaltered parasite. This has been achieved for the myxozoan parasite, *Myxobolus cerebrali.* When Hedrick et al. (2012) exposed fish to UV treated actinospores 63 days prior to their exposure to fully infective parasites, prior exposure resulted in decreased infection prevalence, parasite survival within the host and mature parasite production. Immunization of rainbow trout (*Oncorhynchus mykiss*) to the ciliate *Tetrahymena thermophila* (a less virulent parasite species) prior to another ciliate *Ichthyopthirius multifilus* 6-10 weeks later increased host survival by approximately 50% (Wolf and Markiw 1982). Similarly, Sanchez et al. (2001) demonstrated that rainbow trout exposed to a less virulent strain of *L. salmonae* 15 weeks prior to a more virulent strain resulted in a reduction in xenoma intensity in the gills.

The recent discovery that the myxozoan *Ceratonova shasta* comprises four genotypes (0, I, II and III; Atkinson and Bartholomew 2010a, b) that differ in

virulence presents an opportunity to try to immunize hatchery fish prior to their release. Chinook salmon (*O. tshawytscha*) are commonly infected by both *C. shasta* genotypes I and II, with genotype I causing mortality from enteronecrosis, while infection with genotype II rarely results in mature parasite production or mortality (Hurst and Bartholomew 2012a). Thus, we hypothesize that infection of Chinook salmon with *C. shasta* genotype II, followed by exposure to genotype I, will result in a decrease in disease and/or myxospore production. The association of *C. shasta* with declines in adult Chinook salmon returns (Fujiwara et al. 2011) has focused attention on strategies to reduce parasite abundance and ultimately disease. Thus, immunizing hatchery fish may improve the survival of returning adults by increasing survival of out-migrating juveniles. In addition, a decrease in myxospore production could reduce the overall number of parasites in the river system by reducing transmission to the parasite's next host.

MATERIALS AND METHODS

Parasite Life Cycle

Myxozoan parasites have a complex life cycle involving both an invertebrate and vertebrate host species. For *C. shasta*, the waterborne actinospore stage attaches to and penetrates the gills of a salmonid. Once in the host, the parasite begins proliferating and travels through the blood to the intestine (Bjork and Bartholomew 2010). The parasite then matures into the myxospore stage and is subsequently released into the water column to infect the freshwater polychaete, *Manayunkia speciosa* (Bartholomew et al. 1997).

Exposures

Age 0 Chinook salmon (5-10 g) were obtained from Iron Gate Hatchery (Hornbrook, CA) and transported in aerated coolers to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis, OR (SDL) and held until parasite exposure. A total of 360 fish were randomly placed into four treatments: no parasite (control-treatment 1), genotype II only (treatment 2), genotype I only (treatment 3) and genotype II, then genotype I (treatment 4). Two replicates were used for treatments 1 and 2 as no mortality was expected; treatments 3 and 4 had four

replicates each. All replicates were comprised of 30 fish and exposures were conducted in cylindrical cages of 0.3x1.0 m.

Fish in treatments 2 and 4 were exposed to genotype II for 24 h in the Williamson River, OR (N 42° 32.425, W 121° 52.787) a location where this genotype is predominant and genotype I is absent (Atkinson and Bartholomew 2010a; Hurst et al. 2012b). Water temperature during exposure was 16.5°C. The remaining treatments (1 and 3) were exposed to 18°C UV treated Willamette River water for 24 h at the SDL.

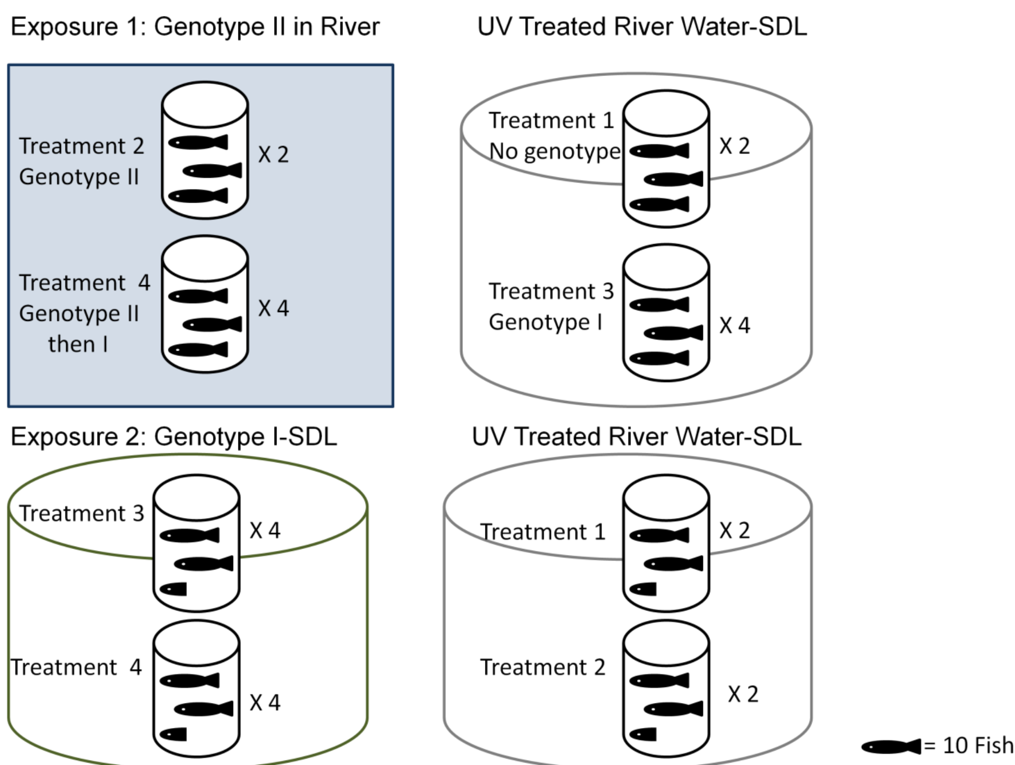

Figure 5.1 Experimental Design. Both exposures were for 24 h with exposure 1 taking place on day 0 and exposure 2 on day 53. Exposure to *Ceratonova shasta* genotype II (exposure 1) took place in the Williamson River, Oregon a known source of genotype II. Exposure 2 took place at the John L. Fryer Salmon Disease Laboratory at Oregon State University, Corvallis, Oregon (SDL). Five fish were euthanized immediately following both exposures for determination of infection.

Exposure to genotype I was conducted in the laboratory where a pure parasite culture could be obtained. The exposure timeline was constrained by the availability of myxospores and polychaetes (Hurst and Bartholomew 2012a) and the activation time required for both the innate and adaptive immune responses (6-12 weeks; Sitja-Bobadilla 2008). Thus, exposure at 53 days after the initial challenge allowed for

activation of both the innate and adaptive immune response and coincided with genotype I production in the laboratory. At the SDL, each treatment replicate was placed in a separate cage within a flow-through 378 L tank containing either genotype I from cultured polychaete populations (treatments 3 and 4) or UV treated Willamette River water (treatments 1 and 2) for 24 h. Water temperatures were 21°C at the time of the second exposure (Fig. 4.1).

Parasite exposure dose

To calculate exposure dose per fish for field and laboratory challenges, 3x1 L of water were collected before and after each exposure. Water samples were filtered, parasite DNA was extracted and parasite density was measured by qPCR for each sample in duplicate (Hallett and Bartholomew 2006). Samples were considered positive if both wells fluoresced and were re-run if a difference of more than one cycle occurred between duplicate wells. One sample from the beginning and end of each exposure was tested for inhibition (Hallett and Bartholomew 2009). If inhibition occurred, samples were diluted 1:10 and re-run. Mean parasite density for each exposure site and time was then multiplied by the velocity and exposure duration and divided by the number of fish (Ray et al. 2010). Velocity was 3 L s^{-1} in the field and 0.0083 L s^{-1} in the laboratory.

Assessment of infection

Immediately after exposure, five fish from each replicate were euthanized with an overdose of tricaine methanesulfonate (MS-222 Argent Chemical Laboratories, Redmond, WA, USA) and the right half of the gill was excised, placed on ice, and stored at -20 °C to determine infection prevalence. The remaining fish were placed into 25 L tanks supplied with well water at 18°C. All fish were treated for external parasites and bacterial infections, fed daily and monitored for clinical disease signs according to Ray et al. (2010). Moribund fish were removed, euthanized as above and time to morbidity was recorded. Myxospores were counted by removing and weighing the intestine, flushing parasites into a microcentrifuge tube using 1 mL of tap water and using a hemocytometer at 200x magnification. A 25 mg aliquot of the harvested myxospores was stored at -20°C for genotype composition (see below). Fish remaining at the end of the experiment were euthanized using an overdose of

MS-222 and a 25 mg piece of intestine was collected and stored at -20°C to determine infection status in survivors (10 fish from each treatment were assayed).

DNA from gills and intestines was extracted and purified as in Hurst et al. (2014). After extraction, DNA from all samples was then directly tested for the presence of parasite DNA using qPCR as above. To create a standard curve for estimating the parasite DNA copy number in 0.1 g of host gill tissue, ten-fold serial dilutions of a synthetic parasite template were added to gill tissue (Hallett and Bartholomew 2006). Harvested myxospores from 10 moribund fish in each of the treatments were sequenced to determine genotype composition (Hurst et al. 2014).

Analyses

Infection prevalence and copy number were determined using gills from treatments 2 and 4 (genotype II; $n = 30$) and treatment 3 (genotype I; $n = 20$) at 1 day post exposure. S-PLUS version 8.2 (Tibco, Palo Alto, CA) was used to compare survival between treatments 3 and 4 and among treatment replicates using a Mantel-Cox test. One replicate from treatment 4 was lost when water flow to the tank was stopped and therefore was not included in the analyses. A one-way ANOVA with Tukey's test for highly significant differences was used to compare natural log transformed (for normality) myxospore counts among treatment replicates. If no differences were detected among replicates within a treatment, replicates were combined for analyses at the treatment level using a student's t-test. Differences were considered significant at $P < 0.05$.

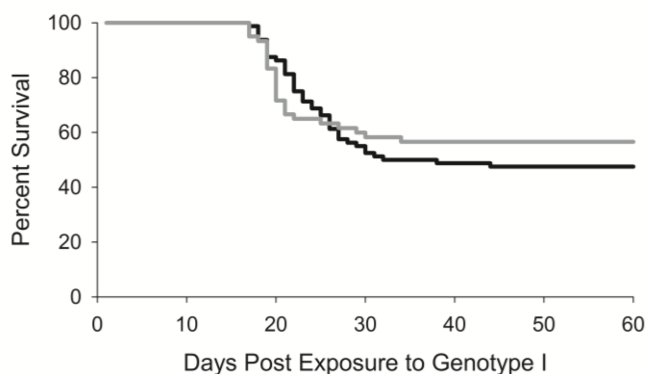

Figure 5.2 Percent survival of Chinook salmon after exposure to *Ceratonova shasta* genotype I only (black line) or after exposure to genotype II followed by genotype I (gray line).

RESULTS AND DISCUSSION

In this study, immunization with a less virulent genotype (II) of *C. shasta* did not prevent or limit mortality or myxospore production in Chinook salmon after a subsequent exposure to a more virulent genotype (I). There was no significant difference in survival between fish exposed to genotype II followed by genotype I (treatment 4; 57%) and genotype I only (treatment 3; 48%; CPH $test_1 = 2.05$, $p = 0.15$; Fig. 5.2). There was also no difference in intestinal myxospore counts between these treatments (t-test, $t_5 = 0.462$, $p = 0.663$), with mean ± SE counts of 8173 ± 2108

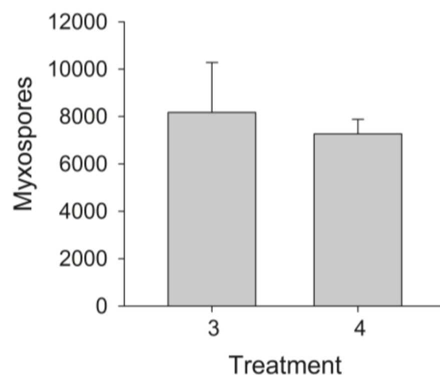

Figure 5.3 Mean number of *Ceratonova shasta* myxospores produced in 0.1 g of intestinal tissue from fish in treatments 3 (exposure to genotype I only) and 4 (exposure to genotype II then I). Error bars indicate standard error.

and 7267 ± 612, respectively (Fig. 5.3). Sequencing demonstrated that myxospores from both treatments were only of genotype I. Infection in surviving fish from treatments 3 and 4 was 50 and 40%, respectively. None of the fish in treatments 1 and 2 died and parasite DNA was not detected in these fish at the end of the study. These data indicate that prior exposure to genotype II does not reduce disease in Chinook salmon. If genotype II was protective, we would have expected at least 74% survival [the 50% infected with genotype II (45/90) in addition to 48% of the remaining naïve fish exposed to genotype I (22/45)].

Differences in exposure conditions and in genotype virulence resulted in variations in exposure dose and infection prevalence. The exposure dose of parasite genotype II in the field was approximately 1.1×10^4 actinospores $fish^{-1}$ and 50% of the fish became infected, as determined by detection of parasite DNA in fish gills. Mean parasite copy number at 24 h was 4.3 (SE, 1.8) using our standard curve for gill tissue ($y = -3.35x + 38.40$; $r^2 = 0.997$). Parasite genotype I exposure dose in the laboratory was lower, 9.2×10^1 actinospores $fish^{-1}$, but resulted in a higher infection prevalence (100%) and a higher mean parasite copy number of 6.7×10^2 (SE, 48.5). Parasite DNA was not detected in the gills of control fish. The faster replication rate of genotype I (author's unpublished data) combined with more optimal flow and temperature conditions for the parasite in the slower flow of the laboratory challenge tanks (Ray and Bartholomew 2013) likely contributed to the higher prevalence and intensity of genotype I infections.

Although these results suggest that immunization using a low virulence genotype is not effective, modifications to exposure timing may yield different results. It is unknown whether the adaptive response plays a protective role in the fish host's defense against *C. shasta*; however, researchers found that rainbow trout infected with a less virulent parasite genotype of *C. shasta* and surviving to three months had a 700 fold increase in IgT antibody levels compared to unexposed fish (Zhang et al. 2010). Work with other myxozoans indicated that specific antibodies were produced from 50 to 360 days post re-exposure by turbot (*Psetta maxima*) in response to *E. scophthalmi* infection (Sitja-Bobadilla et al. 2007) and as early as 35 days post exposure in rainbow trout infected with *M. cerebralis* (Hedrick et al. 1998).

Thus, it seems likely that the adaptive immune response to *C. shasta* infection would have been mounted during our experimental time frame, but a longer time between exposures may have allowed for increased production of a putative protective antibody.

The timing of the subsequent exposure to the more virulent genotype should also consider the timing of the hosts' innate immune response to the parasite, which may be elicited within hours to days of infection depending on temperature and stress (Sitja-Bobadilla 2008; Gomez et al. 2014). For example, infections with *M. cerebralis* resulted in upregulation of immune relevant genes as early as five minutes post exposure (Severin and El-Matbouli 2007). Recently, Bjork et al. (2014) demonstrated that an inflammatory response to *C. shasta* is mounted within at least two weeks of exposure and is capable of resolving infection by 90 days. However, sampling was not conducted between the 25 and 90 day sample times, thus the infection may have been resolved sooner. This suggests that in this study infection with genotype II may have been resolved before exposure to genotype I occurred. A decrease in the interval between exposures may provide some short-term protection for the fish by taking advantage of the mounted inflammatory response.

The lack of protection after initial exposure to genotype II could be attributed to a parasite dose that did not elicit a host immune response and could be rectified by exposing fish to a higher dose of genotype II or lengthening the exposure time. Studies with *T. bryosalmonae* and *E. scopthalmi* demonstrated resistance to parasite reinfection only after lengthy continuous parasite exposures of 10 and 13 months, respectively (Foott and Hedrick 1987; Sitja-Bobadilla et al. 2007). Alternatively, immunization may be parasite genotype-specific, requiring initial exposure to the more virulent genotype to elicit an effective immune response. However, prior exposure to the more virulent genotype increases the probability of fish developing clinical disease. To minimize this risk, fish could be exposed to a low dose of genotype I that enables fish to resolve the infection (Bjork et al. 2014). Despite the difficulties of working with a parasite that has a complex life cycle, immunization studies with *C. shasta* are worth pursuing because they may improve our understanding of how to implement management actions such as dam removal and/or

fish reintroduction in a manner that could provide fish with some level of natural protection against the parasite.

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CHAPTER 6: CONCLUSIONS

SUMMARY

Transmission and persistence of *Ceratomyxa shasta* genotypes-Ch. 2

- Mixed infections are common (83.6%) when fish are naturally exposed to both genotypes I and II.
- Genotype persistence was dependent on exposure dose, infection outcome (mortality or survival) and infection type (mixed or single). High parasite doses led to high host mortality and the persistence of only genotype I, while low parasite doses led to low host mortality and the persistence of both genotypes.

Linking within-host competition to parasite dynamics in a river system-Ch. 3

- In single infections, genotype I replicated faster than genotype II, but caused high host mortality and had lower myxospore production than genotype II, suggesting that genotype II had higher fitness.
- In mixed infections, the total replication rate, myxospore production and host mortality were similar to genotype I alone, but the genotype specific myxospore production for genotype II was reduced, suggestive of competition.
- Production of myxospores by both genotypes suggests both genotypes can coexist within the same host.
- Infections with genotype I first had similar replication, myxospore production and host mortality as concurrent mixed infections.
- When genotype II was afforded prior residency, competitive suppression of genotype II was eliminated. This indicates that genotype II may be able to increase its myxospore production rate in the presence of a competitor to compensate for the decreased production time.

What can cytokine expression reveal about the ecology of parasite genotype interactions?-Ch. 4

- Infections with only genotype I resulted in a delayed host immune response to *C. shasta* that coincided with high inflammatory and regulatory cytokine

expression systemically. This high regulatory expression may have worked to downregulate the inflammatory response before it could prove effective leading to immunosuppression.

- Genotype II infections yielded high inflammatory cytokine expression locally that effectively eliminated infections in 50% of fish.
- Analysis of cytokine expression in mixed infections showed elicitation of both genotype-specific responses. Thus, the suppression of genotype II can be explained by a partially effective immune response locally, while the high virulence of genotype I can be attributed to the immunosuppressive systemic response elicited by genotype I.
- Our results demonstrated that there is immune-mediated competition between genotypes, but it is possible that resource limitation and/or direct interference could also regulate within-host interactions in this system.

Immunization of Chinook salmon against *Ceratonova shasta* using a less virulent parasite genotype-Ch. 5

- Immunization with the less virulent genotype II is unlikely, as we observed no differences in host mortality or myxospore production between immunized and naive fish.
- The lack of immunization may be due to the specificity of the immune response to each parasite genotype.

Synthesis

The high prevalence of mixed infections in naturally exposed Chinook salmon provided the rationale for investigating within-host competition. Extrapolating the results of our experiment to our observations in the Klamath River we were able to suggest an explanation for the current parasite dynamics in the system. The dominance of genotype I in the Klamath system has been attributed to the predominance of Chinook salmon compared to other salmonid hosts. The conclusions from previous field studies were that Chinook salmon were harboring only genotype I, whereas coho salmon were responsible for the minor amounts of genotype II. Our study demonstrates that both genotypes I and II can be produced by Chinook salmon, although genotype I dominates unless genotype II infects fish first.

One important difference between our laboratory results and field studies is that the strain of Chinook salmon utilized differs in its resistance to the parasite. For our laboratory study, the Chinook strain used was more susceptible to infection. This difference in susceptibility is likely what allows both parasite genotypes to produce mature parasites in the host strain used in the laboratory. However, this is still relevant to the natural system because adults returning to the river to spawn are known to be immunocompromised (Robertson 1961). This increases their susceptibility to infection and thus could mean this life stage is responsible for the production of both genotypes I and II. In contrast, the resistant strain of Chinook salmon used in field studies only harbors genotype I. In this case, within-host competition allows genotype I to exclude genotype II before it produces myxospores, leaving only genotype I associated with infection when sampling occurs after fish succumb to infection. Therefore, we suggest that the adult Chinook salmon supplement the genotype II contributed by coho salmon in the Klamath system and that genotype I will remain dominant as long as Chinook salmon are the predominant species.

Our competition study combined with the results of the host immune response during single and mixed infections aids in explaining differences in genotype fitness and virulence. The faster replication rate of genotype I could be explained by the systemic immunosuppression elicited by genotype I. This suggests that the high virulence of genotype I is likely due to the parasite and is not inadvertently caused by excessive inflammation by the host. The effectiveness of the local inflammation elicited by genotype II demonstrates why virulence was lower in fish infected with this genotype. The competitive suppression of only genotype II during mixed infections is likely because the primarily local response to genotype II is ineffective against genotype I. This may be coupled with a limited myxospore production time available for genotype II due to high virulence in mixed infections compared with infections with genotype II alone. The elicitation of genotype-specific immune responses also provides an explanation as to why immunizing fish against genotype I with genotype II was not successful.

FUTURE DIRECTIONS

Within-host competition

Our competition study was conducted in the laboratory in order to achieve parasite doses that would result in some mortality, as host mortality appears to be necessary for myxospore production. However, to test the hypothesis that genotype competition in resistant salmonids results in superinfection versus the co-existence observed in susceptible fish, laboratory studies where both dose and genotype are controlled should be conducted with the Iron Gate strain of Chinook salmon. However, the infectious dose for both genotypes in this host strain is unknown and needs to be assessed prior to beginning the study in order to ensure infection.

To support our hypothesis that the adult stage of Chinook salmon is responsible for maintaining genotype II in the river when the density of other suitable hosts, such as coho salmon, is low, surveys of adult fish are needed. The USFWS surveys adult fish annually and performs myxospore counts. Samples from fish with high myxospore counts should be assayed to determine if they are also the ones with mixed genotype infections. It may also be worth sampling Trinity River Chinook salmon, which resides lower in the system and has less exposure to the parasite. The adults from this strain tend to have a higher prevalence of mixed infections than Iron Gate adults and perhaps the juvenile stage is also more susceptible to genotype II infection.

Host immune response to infection

To further understand the host immune response to *C. shasta* infections collection of the liver in addition to the spleen and intestine could be useful. While the spleen can be used as an indicator of a systemic response, the liver allows assay of acute phase response proteins, such as those involved with complement. In addition, the liver is more appropriate for measuring reactive oxygen species such as nitric oxide produced by cells during infection because of the liver's involvement in filtering toxins from the blood. While qPCR is useful for measuring levels of specific genes of interest, it can be time-consuming and expensive to use this technique as a screening tool for determining genes of interest. Therefore, it may be useful to try looking for differences in gene expression using suppressive subtractive hybridization (SSH).

This technique does not require knowledge of the gene of interest ahead of time, but instead can aid in determining what differences in gene expression exist between a treatment and a control. A brief outline of the SSH technique is as follows:

- Extract mRNA from a control and a treatment sample
- Convert mRNA from the control sample to cDNA.
- Allow the mRNA from the treatment to hybridize with the control cDNA
- Subtract hybridized mRNA and convert unhybrized mRNA to cDNA

The remaining cDNA indicates differences between genes expressed in the control and treatment. Primers can then be designed from the cDNA and expression differences can be quantified using qPCR. However, primer design can become complicated if the region of interest is variable or if the sequence for the gene of interest is novel; significant time should be budgeted.

Host immunization against infection

Although we did demonstrate that immunization with the less virulent genotype II does not protect Chinook salmon from disease after subsequent infections, many immunization strategies remain to be tested. For example, exposure to a non-lethal dose of a more virulent genotype followed by a lethal exposure dose or attenuating actinospores using UV-irradiation might prevent/reduce disease. In addition conducting the re-exposure earlier than the 53 days chosen for our study may provide some protection via innate immunity.

TIPS FOR STUDENTS

This section is a compilation of advice others have passed down and what I myself have learned while working on my PhD.

- If you make your project about a topic you are really interested in, you might still be able to tolerate it by the end
- Outline the chapters of your dissertation as soon as possible
- Before you begin any project whether it is for your thesis or not, be clear about who potential co-author’s are and their role in the study
- Try to focus on only one hypothesis per paper (I did not really learn this until later obviously)

- Dedicate a little time everyday to writing
- Aim to have drafts of your chapters completed a few months before the defense; you will want time to study and put together a clear and concise presentation
- Question everything, even from supposed experts and get multiple opinions before you decide on hypotheses and/or methods
- Conduct a thorough literature review before settling on sampling, supplies and/or techniques
- If you are using samples/data from someone else, make sure they took good notes and you understand the rationale behind their study
- Likewise, be sure to understand the sample history (e.g. has it undergone multiple freezer failures), that way if something doesn't work (e.g. no DNA after DNA extraction), you have a good idea why
- Taking a few days off here and there for mental health is always a good idea

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