Factors Affecting the *Ceratomyxa shasta* Infectious Cycle and Transmission between Polychaete and Salmonid Hosts

AN ABSTRACT OF THE DISSERTATION OF

Sarah J. Bjork for the degree of Doctor of Philosophy in Microbiology presented on March 9, 2010.

Title: Factors Affecting the *Ceratomyxa shasta* Infectious Cycle and Transmission between Polychaete and Salmonid Hosts

Abstract approved: _____

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Ceratomyxa shasta is a myxozoan parasite that infects salmonid fishes causing the disease ceratomyxosis that is characterized by severe hemorrhage and necrosis of the intestine and death of the fish host. *Ceratomyxa shasta* is endemic to the Pacific Northwestern United States and Canada, where epizootics are reported for both wild and hatchery reared fish. Identification of factors affecting the infectious cycle of *C. shasta* is complicated by its obligate two host lifecycle. The parasite infects the fish as an actinospore and develops into a myxospore in the fish host. The myxospore infects the freshwater polychaete, *Manayunkia speciosa* where the actinospore develops. A system for maintaining and infecting *M. speciosa* in the laboratory was developed and a series of laboratory studies tested the effects of temperature on polychaete survival, actinospore release and longevity. Temperature did not affect polychaete survival, but actinospore release occurred earlier and in greater abundance at the higher temperature, whereas actinospore longevity and temperature had an inverse relationship. A laboratory flow experiment tested the effects of two flow rates on *M. speciosa* survival, infection prevalence and fish infection. Polychaetes had higher survival at the fast flow with low infection prevalences compared to polychaetes held in the slow flow treatment. Susceptible rainbow trout became infected when exposed to just 1 actinospore per fish. Fatal infections in these fish were documented at 5 actinospores per fish. Infection prevalence and mean day to death increased with increasing actinospore dose. Fish size did not affect the infective dose; however, parasite dilution did have an effect.

Actinospores were labeled with a fluorescent stain and *C. shasta* attachment to the gills was identified. *In situ* hybridization of histological sections was used to locate the parasite as it migrated from the gill epithelium, to proliferation in the blood vessel, and migration to the intestine. Quantitative PCR was used to quantify the abundance of the parasite in the blood. When the infection of susceptible and Resistant Chinook salmon were compared, there were no differences in actinospore penetration into the gills but resistant Chinook salmon did eliminate parasites in the blood after 2 weeks and isolate parasites in foci of inflammation in the intestine. Resistant Chinook salmon more effectively regulated an immune response to infection, effectively cleared the parasite showed evidence of recovery after infection. The research presented here has been fundamental in performing *C. shasta* infection studies in the laboratory in the fish and polychaete hosts. It has greatly affected our understanding of host parasite interactions including the infective dose for rainbow trout and the recognition of the gills and blood as early sites of *C. shasta* infection. It has also revealed differences in fish host response to infection including the characterization of a recovery from *C. shasta* infection.

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by
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A DISSERTATION

Submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Doctor of Philosophy

Presented March 9, 2010
Commencement June 2010

Doctor of Philosophy dissertation of Sarah J. Bjork presented on March 9, 2010.

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

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ACKNOWLEDGMENTS

I sincerely thank all who have supported me through this process, especially my husband, sister, parents, family and friends. Thank you to my advisor, Jerri Bartholomew, for providing encouragement, guidance, and numerous editorial comments. Thank you to all of my lab mates who put up with a lot; to everyone at the SDL for also putting up with my requests for changes in tank sizes, worm water supply and generally listening to me gripe about worms. Thank you to everyone in the ODFW Fish Pathology lab and the Kent lab for letting me borrow supplies in a pinch and also being supportive. I would also like to thank all members of my committee for providing feedback along the way that has kept me on track.

CONTRIBUTION OF AUTHORS

Jerri L. Bartholomew served as major advisor and contributed to all research papers. Yong-An Zhang and J. Oriol Sunyer designed the primers for the Chinook cytokine expression study and contributed to the interpretation of the results.

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CHAPTER 1: INTRODUCTION

HISTORY

Ceratomyxa shasta is a myxosporean parasite that infects the intestine of salmon and trout and can cause ceratomyxosis, a disease characterized by hemorrhage, necrotic lesions and death. Myxosporeans are a class of the phylum Myxozoa, which are microscopic metazoan parasites (Lom and Dyková, 1992; Kent et al., 2001). The parasite was first described infecting the alimentary tract of farmed rainbow trout (*Oncorhynchus mykiss*) at Crystal Lake Hatchery, CA in 1948 (Wales and Wolf, 1955) and was characterized by Noble (1950). Early research determined that infections were not transmitted horizontally or vertically between fish (Schafer, 1968; Fryer and Sanders, 1970). Although *C. shasta* myxospores were easily identified in the intestine of infected fish, the infective actinospore was not identified until 1997. Bartholomew et al. (1997) identified *Manayunkia speciosa*, a freshwater polychaete as the obligate annelid host and completed the *C. shasta* life cycle by infecting fish with actinospores collected from *M. speciosa* (Figure 1.1).

Ceratomyxa shasta is endemic to the Pacific Northwest (Ratliff, 1983; Ching and Munday, 1984; Hoffmaster et al., 1988; Hendrickson et al., 1989) and infects both wild and hatchery reared fish. Some strains of fish have increased resistance to the parasite and this trait is inherited (Zinn et al., 1977; Ibarra et al., 1992, 1994; Bartholomew, 1998; Bartholomew et al., 2001). However, even inherently resistant fish can succumb to disease with increased exposure to the parasite (Ratliff, 1981; Stocking et al., 2006; Foott et al., 2007a), or at increased water temperatures (Udey et al., 1975b). Evidence of this is seen in the Klamath River where fatal infections in juvenile Chinook salmon (*Oncorhynchus tshawytscha*) (a stock that is considered relatively resistant) are widely reported (Foott et al., 1999; Foott et al., 2002; Foott et al., 2004; Stocking et al., 2006; Foott et al., 2007a; Foott et al., 2007b). The Klamath River was once the third largest Chinook salmon fishery in the U.S. (Graf et al., 2008), but due to low numbers of returning adults, the fishery has been closed in recent years. There is great interest in mitigating the effects of the parasite in effort to enhance the recovery of this recreational, commercial and tribal fishery. One method for lessening the impact of this parasite is interrupting the parasite life cycle. The

complex life cycle of *C. shasta* offers several options to pursue this strategy; but a better understanding of host-parasite interactions are needed to develop effective strategies. Knowledge of factors affecting parasite transmission and resulting infection in both hosts, as well as the criteria for successful fish host defense against the parasite, would identify critical points on which to focus management strategies.

PARASITE LIFE CYCLE AND ECOLOGY

The *C. shasta* life cycle consists of two morphologically distinct parasite life stages occurring in two different hosts (Bartholomew et al., 1997). The myxospore is released from an infected fish and infects the definitive host, *M. speciosa*, in which the parasite develops into an actinospore. The actinospore is released into the water and is infectious for fish.

Myxospore: The myxospore develops in the lower intestine of fish, although development in other organs (kidney, blood, gill, liver, spleen, eye, stomach, pyloric caeca) is common in severe infections (Noble, 1950; Schafer, 1968; Johnson, 1975; Bartholomew et al., 1989). Myxospores are released into the water and infect the polychaete, *M. speciosa*. Knowledge of the viability of the myxospore is limited to the studies of Fendrick (1980), who determined that the morphology of a myxospore remains intact for over 200 days when kept at temperatures of 10°C or below. However, at the time of that study it was unknown if these aged myxospores were infectious, as the alternate host had not yet been identified.

Manayunkia speciosa: Since the elucidation of the polychaete's role in the *C. shasta* life cycle, studies on *M. speciosa* have expanded beyond the morphology and general description of the polychaete, to studies of its habitat, distribution and role in completing the parasite life cycle. *Manayunkia speciosa* was first described by Leidy (1858) and is identified in numerous fresh water benthic surveys across the northern half of North America (Pettibone, 1953; Hazel, 1966; Mackie and Qadri, 1971; Holmquist, 1973). The habitat of *M. speciosa* is characterized by moderate water flows (<0.01 to 3.0 m/s) and fine benthic organic matter (Mackie and Qadri, 1971; Stocking and Bartholomew, 2007). *Manayunkia speciosa* lives at a variety of water temperatures (4 to > 25°C, although the upper temperature threshold is unknown) from Alaskan lakes to California rivers, water flows, and amidst a variety of benthic

substrates. The route of *C. shasta* infection in *M. speciosa* has been described (Meaders and Hendrickson, 2009). Myxospores are ingested by *M. speciosa*, and then the parasite penetrates the gut epithelium of the worm and migrates along peripheral nerves to the epidermis. *Ceratomyxa shasta* undergoes schizogony, gametogony, gametogamy and sporogony in the epidermis of *M. speciosa* (Meaders and Hendrickson, 2009). Bartholomew et al. (1997) speculated that actinospore were released through a secretory pore and the recent study reports the release of actinospores directly through the cuticle by osmotic pressure (Meaders and Hendrickson, 2009).

Much about the biology of *M. speciosa* is unknown and factors that influence the survival of this host would directly affect the distribution and establishment of *C. shasta.* One of the barriers to studying the polychaete has been the inability to establish laboratory cultures. Methods for *M. speciosa* propagation and infection with *C. shasta* in the laboratory were developed during the course of this study (Chapters 2, 3, and 4) and enabled some of these research questions to be addressed. The effect of water temperature and velocity on *M. speciosa* survival and ability to persist was investigated in this study (Chapter 2 and 3). The effect of water velocity on the infection of *M. speciosa* was also investigated (Chapter 3). Additionally, factors affecting actinospore release was unknown and observations on parasite release from live *M. speciosa* is described here (Chapter 3).

Actinospores: Once the actinospore has been released into the water, little is known about its viability and infectivity. The viability of actinospores of other myxosporeans, such as the triactinomyxon of *M. cerebralis*, are affected by time and temperature (Markiw, 1992a; El-Matbouli et al., 1999c; Kerans et al., 2005). Johnson (1975), Ratliff (1983) and Foot et al. (2007b) found a decrease in fish infection prevalence with *C. shasta* when infectious water was held for increased periods of time, thus indicating a decrease in actinospore viability. The relationship between *C. shasta* actinospore age and viability is investigated in the current study (Chapter 2). Other physical parameters such as water flow may influence the physical integrity of the actinospore, as was suggested for the actinospore of *M. cerebralis* (Kerans and Zale, 2002). Water volume and velocity will also affect the concentration and

distribution of the actinospore as well as affect the interaction between actinospore and fish host, as has been demonstrated for *M. cerebralis* (Hallett and Bartholomew, 2008). The effect of water velocity on fish infection severity, infectious dose and how the infectious dose is affected by actinospore concentration is reported (Chapters 2 and 3).

Salmonid host: Prior to this study, the route of *C. shasta* actinospore invasion into the fish host was unknown. A fluorescent cytoplasmic stain was used to label actinospores and the site of invasion was identified (Chapter 5). *In situ* hybridization was used to follow the migration of the parasite from the site of invasion to the intestine (Chapter 5).

In the fish host, the severity of *C. shasta* infection depends upon the inherited resistance genetics of the fish, genotype of the parasite, parasite dose and environmental conditions. Although the relative susceptibility of different stocks has been established (Zinn et al., 1977; Buchanan and Sanders, 1983; Ching, 1984; Ching and Munday, 1984a; Ibarra et al., 1991a; Bartholomew, 1998), identification of an infective dose for fish has been complicated. Infection has been induced in the laboratory by injecting rainbow trout, cutthroat trout (*Oncorhynchus clarki*), brown trout (*Salmo trutta*), Atlantic salmon (*Salmo salar*) and susceptible fall Chinook salmon with trophozoites (Johnson, 1975; Ibarra et al., 1991). Using a natural exposure method, Foott et al. (2007a) calculated that over 300,000 actinospores delivered over a 3 h corresponded to 37% infection in a resistant strain of Chinook salmon, however, interpretation of these results in terms of infective dose is confounded by water temperature and velocity. Prior to the elucidation of the life cycle and discovery of the actinospore, Ratliff (1983) determined that one infectious *C. shasta* parasite was capable of inducing lethal infection in susceptible rainbow trout. Here, we expand on that observation with controlled challenges and examination of the effect of dose on disease severity (Chapter 3).

Some of the differences in fish infection may come from genetic differences in the parasite itself. Atkinson and Bartholomew (in press) have identified unique genetic sequences in *C. shasta* isolated from different fish host species. The fate of any given parasite genotype in a fish host is unknown; however these differences may

explain the variability in fish infection between species challenged with the same concentration of *C. shasta* actinospores (Hallett and Bartholomew, 2006; Stocking et al., 2006).

Regardless of parasite genotype strain, stocks within the same species (*O. mykiss, O. tshawytscha,* or *O. kisutch*) show different susceptibility to *C. shasta* (Zinn et al., 1977; Buchanan and Sanders, 1983; Hemmingsen et al., 1986; Ibarra et al., 1991), and resistance traits are inherited and conferred on multiple loci (Ibarra et al., 1992; Bartholomew et al., 2001; Nichols et al., 2003). The mechanisms of *C. shasta* resistance are unknown, but it has been hypothesized that: 1) there is resistance at the site of entry, 2) parasites are prevented from proliferating and establishing in the intestine and 3) resistant fish develop an effective immune response (Ratliff, 1981; Bartholomew et al., 1989; Ibarra et al., 1991a; Ibarra et al., 1994). Even "resistant" fish succumb to infection when parasite dose is sufficiently high; therefore, there is a dose dependent threshold for which these defenses are effective. In this study, the role of each of these defense strategies and their threshold relative to parasite dose is investigated (Chapter 6).

FISH HOST RESPONSE TO INFECTION

All jawed vertebrates have both an innate and adaptive immune system (Kasahara et al., 2003). The processes of the immune response (both innate and adaptive) has been most simply described as recognition, activation and differentiation (Thaler et al., 1977). The overall function of salmonid fishes' immune response is analogous to what occurs in mammals and birds, yet as poikilothermic animals the adaptive immune response occurs much slower and many of the cells involved and mechanics of the pathways have yet to be characterized.

Innate Immune Response: All invading pathogens of fish must surpass the skin, mucosal epithelium, and mucus that act as the primary physical barrier to infection and covers virtually all body surfaces in contact with the environment (Ingram, 1980; Shephard, 1994; Ellis, 2001; Magnadottir, 2006; Whyte, 2007). The mucus acts to trap, lyse or slough off pathogens and contains numerous acellular non-specific immune components. Lectins, complement proteins, pentraxins, and hemagglutins tag pathogens as foreign agents and lead to phagocytosis; lysozyme

destroys pathogens through enzymatic lysis. The mucus and skin also contain histone-like proteins that inhibit parasite development (Noga et al., 2002). Precipitins (Cipriano and Heartwell, 1986), proteases (Hjielmeland et al., 1983), and apolipoproteins (Villarroel et al., 2007) also act to trap, immobilize or lyse invading pathogens (Ingram, 1980; Jones, 2001). Natural (non-specific) antibodies and pathogen specific antibodies produced by the adaptive immune system are also present in the mucus (Magnadottir, 2006). Pathogens that evade mucus defenses would then encounter the skin, a physical barrier composed of tight junctions between cells that also contains innate immune cells such as macrophages, eosinophilic granular cells and rodlet cells, as well as numerous humoral factors (Magnadottir, 2006; Whyte, 2007).

Once the pathogen has surpassed the mucus and epithelium, the host response to infection depends upon the nature of the pathogen (bacterial, viral, fungal, protozoan, or metazoan), the pathogen's site of invasion, its target tissue, and its migration route to reach this tissue. Hence, the progression of an immune response to a pathogen that enters through the digestive tract would be different from a pathogen that penetrates the dermis and muscle or one that enters through the gills and migrates into the blood stream. Once a pathogen enters the tissue, the immune response is initiated by host recognition of the pathogen by resident innate immune cells or humoral factors. The host's ability to recognize the pathogen as foreign would depend on the host's germline encoded pattern recognition receptors (PRRs) and the pathogen's surface antigens (Whyte, 2007). If the host recognizes pathogen associated molecular patterns (PAMPs) on the surface of the pathogen, or damaged host cell antigen produced by intracellular invasion of or damage to the host cells, the pathogen would be phagocytosed or targeted by the humoral components previously described. Once the pathogen is recognized, signal transduction and gene expression would be initiated to produce cytokines and chemokines to recruit phagocytic cells, granulocytes, other lymphocytes, and humoral factors such as complement to the site of infection and generate inflammation (Bayne and Gerwick, 2001; Whyte, 2007). Monocytes (such as macrophages) phagocytize the pathogen or infected host cells, and present pathogen specific antigen to T and B cells activating the adaptive immune

response (below). Meanwhile, humoral factors such as C3 and other opsonins continue tagging the parasite and other courses of pathogen destruction such as the complement pathway, induced nitric oxide synthase (iNOS), or lysozyme, all act to lyse the pathogen (Holland and Lambris, 2002; Magnadottir, 2006; Whyte, 2007). Through these means, inflammation acts to both trap and neutralize the pathogen.

Adaptive immune response: As these innate immune processes are occurring *in situ* with the pathogen, the adaptive immune response is being stimulated by pathways. Complement bound to pathogens, antigen presenting cells, as well as cytokines and chemokines that are released from activated cells initiate differentiation of T and B cells in the head kidney and spleen. The type of response and the cells involved: T- helper 1 (Th1), T-helper 2 (Th2) or T-regulatory (T_R), is determined by the antigen presenting cell functions and the cytokines expressed (Jankovic et al., 2001; Secombes et al., 2009). Th1 responses are involved in delayed type hypersensitivity responses and intracellular pathogens such as viruses, while Th2 responses are involved in defense against extracelluar parasites (Secombes et al., 2009). The T_R pathway inhibits the activation of other T-cells and innate immune cells (Secombes et al., 2009). Upon stimulation by the appropriate cytokine, the corresponding T-cell sub-set proliferates and produces more cytokines further tailoring the response to the pathogen. Depending on the presentation of the antigen (via MHC I or II) and co-stimulatory molecules (CD4+ or 8+), cell mediated cytotoxicity may proceed to destroy the infected or damaged host cells (Fischer et al., 2006). Alternatively, leukocytes presenting pathogen specific antigen circulate to the spleen and head kidney and stimulate B cells to differentiate, resulting in clonal expansion of plasmablasts and plasma cells which produce antibodies (immunoglobulins, Ig) specific to the pathogen (Kaattari et al., 2009). Fish produce 4 classes of antibody: IgM, IgD, IgT, and IgZ (Kaattari et al., 2009). Antibody production is facilitated by expression of RAG (recombination activating gene) which controls the diverse components of antibodies through VDJ recombination (Kasahara et al., 2003; Klein and Nikolaidis, 2005). As the infection and immune response continues, antibodies develop higher affinity for the antigen (Voss et al., 1978; Kaatari et al., 2002). Antibodies and antibody secreting cells leave the spleen and

head kidney and circulate and localize to the infection (Bermudez et al., 2006). Antibodies bind to the antigen via the light chain of the immunoglobulin, and permit recognition of the pathogen by effector cells through binding to the heavy chain. Clearance of the pathogen proceeds by phagocytosis or lysis. Antibody secreting cells home back to the head kidney and may be maintained for extended periods of time as memory cells (Kaatari et al., 2005). The duration of immunological memory is highly dependent on temperature; for example, in carp specific Ig was detected up to 70 days after initial infection, with the peak occurring around 4 weeks after antigen exposure (Avtalion, 1969). An enhanced secondary response also occurs in rainbow trout, in which a higher antibody titer is reached upon second exposure to the antigen (Arkoosh and Kaatari, 1991).

Fish host response to myxozoan infections: The host response to myxozoan infection depends on the nature of the parasite. There are several reviews that discuss the innate and adaptive immune response to parasitic and myxozoan infections in particular (Woo, 1992; Jones, 2001; Sitjà-Bobadilla, 2008). Many coelozoic parasites do not induce an immune response in their host, however, innate and/or adaptive immune responses are mounted against some histozoic myxozoans. In some infections, host response to the early stages of infection is lacking and mimicry of host antigens was predicted as a means of evading the host immune system (Pauley, 1974). The alternative complement pathway plays a major role in the elimination of some parasites (Woo, 1992) but cell mediated cytotoxicity (Whyte, 2007) and antibody responses can also be involved (Sitjà-Bobadilla, 2008).

Myxobolus cerebralis, the cause of salmonid whirling disease, is one of the most well studied histozoic myxozoans. This parasite invades the epithelium of its fish host then migrates through the dermis and along the nerves to the cartilage, where it proliferates and causes lesions (Wolf and Markiw, 1984; Markiw, 1989; El-Matbouli et al., 1992; El-Matbouli et al., 1995). As the *M. cerebralis* sporoplasms invade the dermis, lymphocytes and macrophages localize to the infected site in susceptible rainbow trout and parasites that do not reach the nerve within 4 days are destroyed by cellular and humoral responses (Hedrick et al., 1998; El-Matbouli et al., 1999). In the Hofer strain of rainbow trout, a resistant strain from Germany, more

parasites are eliminated in the dermis than in susceptible Trout Lodge strain (Baerwald et al., 2008). These resistant fish express higher amounts of ubiquitin and metallothionin B (Baerwald et al., 2008). Ubiquitin plays a role in proteolysis and could aid in pathogen destruction. Metallothionin B is a transcriptional regulator associated with inflammation, immune response, apoptosis, and detoxification. Thus it appears that the resistant fish up-regulate a broad spectrum of defenses to a greater degree than susceptible fish. The Hofer strain of rainbow trout and brown trout (which also has increased resistance to whirling disease), both have eosinophilic granular cells (EGCs) associated in the root ganglia in response to infection (Hedrick et al., 1999; Baerwald et al., 2008). EGCs are also found around cranial nerves of infected brown trout and coho salmon, another species comparatively resistant to *M. cerebralis* (Hedrick et al., 1998). It is proposed that these cells intercept *M. cerebralis* at the nerve and eliminate the parasite prior to migration to the cartilage.

When *M. cerebralis* reaches the cartilage, the parasite proliferates and causes lesions. Macrophages can be found surrounding these lesions in the cartilage and granulomatous inflammation eventually develops late in the infection (Hedrick et al., 1998). An antibody response to *M. cerebralis* does occur, but it is inconsistent between fish, is not formed against a uniform antigen, and is dose dependent (Hedrick et al., 1998). Fish challenged with parasite doses too low to initiate cartilaginous lesions did not develop immunity, whereas fish challenged with parasite doses high enough to lead to the development of lesions developed active acquired immunity and resisted re-infection even when challenged 35 days later with high parasite doses (Hedrick et al., 1998a).

Proliferative kidney disease is caused by another myxozoan parasite, *Tetracapsuloides bryosalmonae* (Canning et al., 1999) and the immune response of fish to this parasite has also been studied. *Tetracapsuloides bryosalmonae* invades the gill then migrates to the kidney via the blood stream (Smith et al., 1984; Morris et al., 2000). Little is known about the early host response to invasion, but it is an overzealous immune response in the fish that is credited with causing the disease state. Once the parasite localizes to the kidney, the proliferation of lymphocytes in the infected kidney induce lymphoid tissue hyperplasia (MacConnell et al., 1989),

resulting in clinical signs of proliferative kidney disease (Chilmonczyk et al., 2002). Infected fish showed reduced phagocytic activity, reduced oxidative burst by granulocytes (Chilmonczyk et al., 2002) and high production of immunoglobulins (Olesen and Jorgensen, 1986). A specific, protective acquired immune response (Clifton-Hadley et al., 1984; Klontz et al., 1986; Foott and Hedrick, 1987) develops only in fish that had exhibited clinical signs of infection (Foott and Hedrick, 1987).

Enteromyxum scophthalmi is another myxosporean that elicits a protective antibody response in fish hosts that survive initial infection (Sitjà-Bobadilla et al., 2007). Of these examples, *E. scophthalmi* most resembles *C. shasta* in that it infects the intestine, resulting in hemorrhage and necrosis (Branson et al., 1999). *Ceratomyxa shasta* is specific to salmonids whereas *E. scophthalmi* infects turbot, *Scophthalmus maximus*. The route of *E. scophthalmi* invasion in turbot is not yet known, but the infection can be transmitted by cohabitation (Branson et al., 1999; Sitjà-Bobadilla et al., 2004). Early in *E. scophthalmi* infection, the circulating granulocytes and respiratory burst are increased and leukocytes infiltrate into the intestine. Later in infection there is an increase in total serum anti-proteases whereas serum complement and lysozyme are depleted (Sitjà-Bobadilla et al., 2006). Immunoglobulin M (IgM) was found in the of lamina propria of *E. scophthalmi* infected fish (Bermudez et al., 2006), leading Sitjà-Bobadilla et al (2006) to hypothesize that T-lymphocytes are part of the initial cellular response followed by the production of antibodies occurring later in infection. It also appears that antibody (Ig) producing cells are recruited to the intestine (Bermudez et al., 2006). Similar to *T. bryosalmonae* and *M. cerebralis* infections, the acquired immune response (at least based on the number of Ig positive producing cells in the intestine) increased greatly when infection severity increased from light to mild or severe (Bermudez et al., 2006). Thus, fish can develop a protective acquired immune response to myxozoans and it appears that there is a minimum infection intensity required to mount this response.

Recent studies have identified an antibody response to *C. shasta* in susceptible rainbow trout surviving infection (J. Oriol Sunyer, personal communication). Surviving fish had increased serum IgM and IgT in the mucus. Parasites in the lamina propria and trophozoites in the lumen were coated with mucosal antibody. This

response was observed late in the infection (3 months p.i.) and it is not known if these fish would be protected upon re-infection with the *C. shasta*. Thus far, this response has only been investigated in susceptible fish.

Fish host resistance at the site of pathogen invasion: Resistance at the site of entry is a common strategy in fish as a means of protection against pathogens. Increased bactericidal activities were observed in the mucus of coho salmon strains resistant to *Listonella anguillarum* (formerly *Vibrio*), in comparison to more susceptible strains (Iwama and Balfry, 1998). Similarly, antibacterial properties in the mucus are associated with increased resistance to *Aeromonas salmonicida* infection in resistant steelhead and brown trout (Cipriano et al., 1994), and higher levels of mucus precipitin in brown trout is associated with inherited resistance (Cipriano and Heartwell, 1986). In previous studies, observations of immature *C. shasta* trophozoites in the lumen of the intestine of resistant fish which presented several possibilities for resistance, one of which was exclusion from invasion if the parasite entered from the lumen of the intestine (Bartholomew et al., 2004).

Fish that develop acquired immunity to their respective pathogens are also capable of resisting re-invasion. Catfish that survived *Ichtyophthirius multifilis* infection have binding antibody in the mucus that prevents attachment of parasites upon re-infection (Clark et al., 1995; Dickerson and Clark, 1998). The tomato clownfish, *Amphiprion frenatus*, also produces antibody in the skin and gill that is associated with preventing re-infection by the dinoflagellate *Amyloodinium ocellatum*, the cause of marine velvet disease (Cobb et al., 1998). Bighead carp, *Aristichthys nobilis* (Richardson) that survived initial infections with *Lernea polymorpha* either rejected parasites from the skin or limited infection to the skin and prevented invasion deeper into the tissues upon re-exposure (Shariff and Roberts, 1989).

Prevention of proliferation and establishment: It is the goal of both the innate and acquired immune responses to prevent proliferation and establishment of pathogens. Strategies that prevent invasion of the pathogen (see previous) would provide the first line of defense, but if invasion occurs several other defenses can be recruited. In myxozoan infections one or both of the innate and/or adaptive pathways

are involved (see *Fish host response to myxozoan infections*). In injection experiments with *C. shasta* trophozoites (which bypassed the defenses of the skin and mucus), resistant fish isolated invasive trophozoites in granulomas (Ibarra et al., 1991; Foott et al., 2004). These observations lead to the hypothesis that resistant strains of fish prevent proliferation of the parasite and establishment in the intestine. The ability of resistant strains of fish to arrest parasite development would depend on the host's ability to recognize the parasite as foreign and then orchestrate an appropriate response to contain and neutralize the parasite. Differences between susceptible and resistant fish could then depend on: (1) inherently different levels of resident phagocytes or humoral factors which would indicate that resistant fish are more primed for response to infection; (2) different kinetic rates in which lymphocyte proliferation or maturation would occur faster in resistant fish; or (3) earlier recognition of the parasite resulting in a faster and more specific initiation of the immune response overall.

The *C. shasta* host parasite relationship is complex, with a multitude of factors capable of affecting the completion of the life cycle at any host or life stage. In order to develop strategies for parasite control, more information on host-parasite interactions is needed. Understanding the relationship between *C. shasta* actinospore infectivity and changing environmental parameters would increase the ability to predict fish infection severity based on river conditions. Insight into the immune response against *C. shasta* infection will lead to future studies that can determine if fish mount a protective antibody response against *C. shasta.* If fish can mount a protective antibody response against *C. shasta*, management practices could be shifted towards facilitating parasite doses, parasite antigen exposure, water temperatures or water flows that induce this response. Additionally, this information would be pivotal to developing accurate predictive models for the disease.

RESEARCH GOALS

The goal of this research was to identify environmental factors that affected the *C. shasta* infectious cycle and transmission between hosts. The effects of changing environmental parameters on the survival and infection of *M. speciosa* (Chapters 2 and 4), the viability of the actinospore (Chapter 2), and infection in

rainbow trout and Chinook salmon were investigated. Additionally, this research sought to characterize the infection in the fish host by determining the infective dose for susceptible rainbow trout and resistant strains of Chinook and coho salmon (Chapter 5), the route of transmission of actinospores from *M. speciosa* (Chapter 4) to infection of fish (Chapter 6) and the mechanisms utilized by resistant fish to survive infection with this parasite (Chapter 7). Overall, the purpose of this study was to better understand the parameters required for successful transmission of parasite life stages between hosts and to contribute to the ability to make predictions about the severity of fish infection based on environmental parameters. The specific objectives of the study are as follows:

Objective 1) Determine the effect of temperature on the survival of *M. speciosa* infected with *C. shasta,* as well as its effect on actinospore release and on actinospore viability. *Approach*: In the laboratory, a population of *M. speciosa* was infected with *C. shasta* in the fall. During the winter, infected *M. speciosa* were acclimated to 4, 12, or 20°C water and their survival and actinospore release were monitored over a 30 days. Additionally, actinospores collected from infected polychaetes were held at the same experimental temperatures and their viability was determined.

Objective 2) Determine the effect of water velocity on *M. speciosa* survival and the transmission of *C. shasta* between fish and polychaete hosts. *Approach*: A laboratory flow experiment was developed to test the survival of *M. speciosa* and infection prevalence of polychaete populations when exposed to *C. shasta* myxospores when held at either a 0.01 m/s (slow) or a 0.05 m/s (fast) flow. The prevalence and severity of infection in rainbow trout (susceptible strain) and Chinook salmon (resistant strain) challenged with actinospores released from *M. speciosa* at each of the flows were also determined.

Objective 3) Determine the infective dose for susceptible rainbow trout and resistant Chinook and coho salmon. *Approach*: Actinospores were collected from a *M. speciosa* population that was infected in the laboratory and a susceptible strain of

rainbow trout and comparatively resistant strains of Chinook and coho salmon were challenged with controlled actinospore doses. Chinook salmon were challenged at high (18°C) and low (13°C) temperatures and susceptible rainbow trout were challenged at different actinospore concentrations and the effect of fish size on infective dose was also investigated.

Objective 4) Identify the route of *C. shasta* transmission from *M. speciosa* to the fish host. *Approach*: Live *M. speciosa* were monitored for actinospore release. Histology and transmission electron microscopy were also used to identify the structures involved in actinospore release. Fluorescently labeled actinospores, *in situ* hybridization, and quantitative PCR were used to identify *C. shasta* attachment, tissue penetration and migration in infected fish.

Objective 5) Compare and characterize the fish host response of a susceptible and resistant strain of Chinook salmon to *C. shasta*. *Approach:* Use quantitative PCR and *in situ* hybridization to follow the course of a *C. shasta* infection and identify fish tissue and time after infection where parasite development is arrested. Determine if the host response in resistant Chinook salmon is dominated by the Th1 or Th2 pathway. The expression of the pro-inflammatory cytokines indicative of Th1 response: IL-1β, IL-8, TNFα, and IFNγ and the expression of cytokines indicative of Th2: IL-4, IL-6, and IL-10 were compared between infected and non-infected fish of both strains. For further characterization of the immune response T_R cytokines TGFβ and IL-10 were also included.

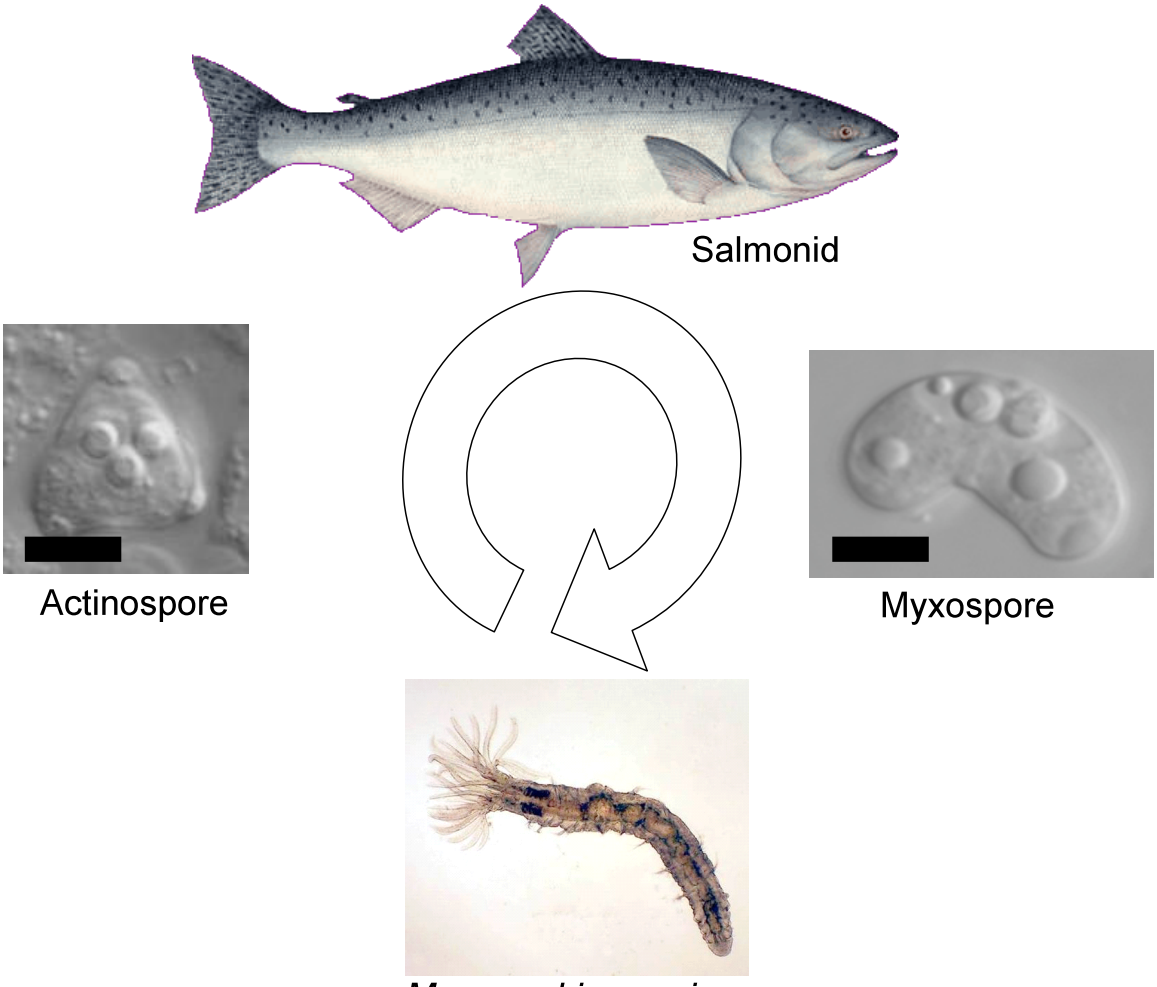

Figure 1.1 *Ceratomyxa shasta* life cycle. Scale bars indicate 5 µm. Actinospores infect the salmonid host and develop in the myxospore in the intestine. Myxospores are released from the intestine into the water column and infect the freshwater polychaete worm *Manayunkia speciosa.*

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CHAPTER 2: EFFECTS OF TEMPERATURE ON THE SURVIVAL OF *MANAYUNKIA SPECIOSA* INFECTED WITH *CERATOMYXA SHASTA*, THE RELEASE OF ACTINOSPORES AND IMPLICATIONS FOR ACTINOSPORE VIABILITY

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For submission to Parasitology

ABSTRACT

The actinospore stage of the myxozoan parasite *Ceratomyxa shasta* is the life stage infectious to fish and develops in the freshwater polychaete *Manayunkia speciosa*. This study investigated the effects of temperature on the survival of infected *M. speciosa*, the rate of actinospore release and the longevity of actinospores at 3 temperatures under laboratory conditions. There were no differences in survival of *M. speciosa* infected with *C. shasta* at 4, 10 or 20ºC. Infected *M. speciosa* acclimated to 20°C released the largest number of actinospores and did so earlier than worms at lower temperatures, as determined by both quantitative molecular methods and visual examination. No actinospores were released from infected *M. speciosa* at 4ºC. There was an inverse relationship between temperature and viability of the actinospore. Actinospores held at 4°C maintained intact morphology up to 18 days after collection, whereas, actinospores held at 20ºC remained intact for only 6 days. Thus, at warmer temperatures, *M. speciosa* infected with *C. shasta* would release a greater number of actinospores but their viability would be lower.

Keywords: *Ceratomyxa shasta*, *Manayunkia speciosa*, temperature, actinospore, viability, polychaete

INTRODUCTION

Manayunkia speciosa is the definitive annelid host of *Ceratomyxa shasta* (Bartholomew et al., 1997), a myxozoan parasite that infects the intestine of salmonids. This small freshwater tube dwelling sabellid polychaete worm has a broad distribution in the benthos of freshwater lakes, streams, and rivers across North America (Mackie and Qadri 1971). Within this range, *M. speciosa* inhabits waters subject to a versatile range of temperatures (from 4 to > 25°C). A recent study (Meaders and Hendrickson, 2009) determined that *M. speciosa* becomes infected by ingestion of myxospores, the parasite stage released from infected fish. The parasite penetrates the intestine, migrates to the dermis and develops into mature actinospores, which when released infect the fish host.

Infection of fish has a seasonal component (Ratliff, 1983; Hendrickson et al., 1989; Margolis et al., 1992) and there is a direct relationship between temperature and infection severity in the fish host (Udey et al., 1975). Less is known about the effect of temperature on the polychaete and actinospore stage of the parasite. However, an inverse relationship between temperature and time on the infectivity of river water containing *C. shasta* is reported (Ratliff, 1983; Foott et al., 2007). In other myxozoan host-parasite relationships temperature is known to affect host survival; the rate of parasite development and release; and parasite survival once released into the aquatic environment. In the *Myxobolus cerebralis* life cycle, the rates of actinospore development (El-Matbouli et al., 1999; Blazer et al., 2003) and release (Kerans et al., 2005) from its annelid host, *Tubifex tubifex*, are affected by temperature. Once released from this host there is an inverse relationship between temperature and actinospore viability (Markiw, 1992; El-Matbouli et al., 1999). Temperature also affects the population growth of *T. tubifex* (Kerans et al., 2005).

To better understand the seasonal dynamics of *C. shasta*, we examined the effect of 3 different temperatures on the survival of *M. speciosa* infected with the parasite, on the rate of actinospore release from *M. speciosa* and the viability of the actinospores once released from this host.

MATERIALS AND METHODS

Collection and maintenance of infected M. speciosa*:* Sediment containing *M. speciosa* was collected from Upper Klamath Lake at the mouth of the Williamson River (GPS: 42º 25.282' N, 121º 55.836' W), in October 2006, using an 83 µm mesh plankton net (17 cm diameter) fitted on a telescoping handle. Material was placed in plastic bags with approximately 500 mL of river water, supplied with oxygen using an airstone and transported to the John L. Fryer Salmon Disease Laboratory (SDL), Oregon State University (OSU), Corvallis, OR, USA. Sediment was transferred to an outdoor tank supplied with a continuous flow (approximately 1L/min) of untreated Willamette River water. Infection in this colony of *M. speciosa* was initiated by adding *C. shasta* myxospores on a weekly to bi-weekly basis for at least 2 months (Bjork and Bartholomew, 2009a). In February 2007, infection was confirmed visually by examining 30 *M. speciosa* for developmental stages of *C. shasta* in the polychaete dermis with the aid of phase contrast light microscopy; *C. shasta* was identified in 24 worms. Five additional worms were collected and tested individually for *C. shasta* DNA by PCR (Stocking and Bartholomew, 2007); all 5 were positive. Water temperature was not controlled in the infected *M. speciosa* colony and changed seasonally from 16ºC in October to 8ºC in February when *M. speciosa* was removed from the colony for initiation of the temperature experiment.

Temperature effects on C. shasta *infected* M. speciosa *survival:* Ninety polychaetes acclimated to 8 ºC were removed from the sediment using modified dental picks and pooled. Efforts were made to leave the polychaete tube intact, and transfer the entire tube with the animal. Polychaetes were randomly selected and randomly assigned one by one into 3 wells of 3 24-well tissue culture plates for a total of 10 per well. Willamette River water was adjusted to 4, 10, or 15 °C and one mL added to each well prior to addition of the polychaetes. Each plate was transferred to an incubator of the appropriate experimental temperature 4, 10, or 15 °C on the first day. On day 2, the temperature of the 15 ºC incubator was increased to 20 ºC, thus providing experimental temperatures of 4, 10 and 20ºC for the remainder of the experiment. Every other day, the water in the wells was gently mixed by aspiration using a pipette, then removed and examined for actinospores (see below). Willamette River water was collected every other day, adjusted to the experimental temperature

(4, 10 or 20ºC), and used to replace the water in each well. After 30 days, polychaetes were removed from the wells and enumerated.

Temperature effects on actinospore release: The water aspirated from the wells during water changes was assessed for actinospores by visual and molecular methods every other day for the first 15 days. Additional visual monitoring of actinospore release was done on day 18 and at termination of the experiment on day 30. A single 1 mL of Willamette River water was assayed in parallel to determine if the water supply contributed any parasite during the experiment.

Quantification of actinospores released: One 5 µL aliquot of water from each well was individually placed on a microscope slide with a coverslip and the actinospores in the entire aliquot enumerated using phase contrast microscopy. The remaining 995µL from each well was aspirated into a syringe and filtered onto a 2 cm diameter 5 µm Millipore filter using a syringe filtration adapter. The filter was frozen until DNA extraction and assay by qPCR (Hallett and Bartholomew, 2006). Each sample was assayed in duplicate wells through 40 cycles in the qPCR assay using an ABI 7300 Real-time PCR system and MicroAmp ® optical 96 well plates. If the standard deviation of the duplicate wells for an individual sample was >1, the sample was assayed again. Two internal positive controls (DNA from an infected fish tissue and an artificial parasite DNA template) and a no template control (NTC, molecular grade water) were included each time the qPCR assay was run. Only samples for which both wells fluoresced were considered positive for parasite DNA. A Cq (quantitative cycle) value of 38 is the threshold for one actinospore, therefore any Cq >38 is less than 1 actinospore. Samples that were undetected were assigned a Cq value of 40 to permit analysis.

Manayunkia speciosa *infection prevalence after 30 days at the experimental temperatures:* To assess infection prevalence of *M. speciosa* at the end of the experiment, all polychaetes were individually frozen then assayed for the presence of parasite DNA by PCR (Stocking and Bartholomew, 2007).

Statistical analysis: All statistical analyses were performed using S Plus version 8.0, Insightful Technologies, Seattle, WA, USA.. One way ANOVA was used to analyze the variance within and between groups to determine if *M. speciosa*

survival at the experimental temperatures were significantly different. Linear regression with interaction between temperature and time was used to determine if the rate of parasite release over time was significantly different at each of the temperatures. One way ANOVA was used to analyze the variance in the infection prevalence of *M. specisoa* within and between groups acclimated to the experimental temperatures.

Temperature effects on C. shasta *actinospore viability:* Actinospores were collected from polychaetes with mature infections and enumerated (Bjork and Bartholomew, 2009a). Actinospores were pooled in approximately 300µL specific pathogen free well water in a microcentrifuge tube, with an initial density of 1,500 actinospores/mL. This volume was divided into six 50 µL aliquots. Two aliquots were placed in incubators set to 4, 12, and 20°C. Every 3 days for 18 days, a single 5µL sample was removed from each tube and actinospores enumerated. Due to only duplicate samples, no statistical analyses were performed.

RESULTS

Temperature effects on C. shasta *infected* M. speciosa *survival:* The average survival was 70% for 4 and 20ºC and 80% for 10ºC and was not significantly different between temperatures (One way ANOVA p = 0.94) (Table 2.1). One of the 20ºC replicates was lost due to handling on day 9.

Temperature effects on actinospore release: Actinospores were not visually detected in any of the temperature groups until day 11, when actinospores were seen in the 2 replicates at 20°C (Table 2.2). Actinospores continued to be detected in the 20°C replicates for the remainder of the 15 day experiment and in samples taken at 18 and 30 days. On day 13, actinospores were first detected in one of the 10°C treatment replicates. Visual detection in each of the other 10°C replicates occurred at least once during the experiment but was variable. Actinospores were detected in the 10 and 20ºC replicates on days 18 and 30. No myxospores were seen in any of the wells at any time and neither parasite stage was observed in any of the Willamette River water samples. No actinospores were observed in any of the 4°C replicates.

By qPCR analysis, Cq values varied between all temperatures over time and there was a significant difference between the rate of actinospore release between

temperatures (Linear regression with interaction between temperature and time $p < 0.001$) (Figure 2.1). The highest amount of *C. shasta* DNA (lowest Cq value) was detected in the 20°C replicates, and the lowest detected in the 4°C replicates. Willamette River water had Cq values greater than 38 for all sampling times, equating to less than one actinospore.

Polychaete infection prevalence after 30 days at the experimental temperatures: Polychaete infection prevalence at the termination of the experiment was between 67 -100% for individual replicates and the average prevalence was between 83-100% (Table 2.1). There is no statistical difference in the prevalence of infection of *M. speciosa* held at the different temperatures (One way ANOVA $p = 0.12$).

Effects of temperature on C. shasta *actinospore viability:* Actinospore numbers declined in all three temperature groups over time (Figure 2.2). Actinospores at 4°C could be detected out to 18 days but declined in number with time. Actinospores at 12 °C had a more intermediate decline and were detected until 15 days after initiation of the experiment. The 20°C treatment group saw the sharpest decline with no actinospores seen after 9 days.

DISCUSSION

Much about environmental factors affecting *C. shasta* infection in *M. speciosa* is unknown and this study was the first to investigate the effects of temperature on polychaete survival, the course of infection, and the viability of the actinospore. Temperature had a direct effect on the maturation and release of *C. shasta* actinospores but did not affect the survival of infected *M. speciosa*. When infected polychaetes were held at warmer temperatures, actinospores were released earlier and in higher abundance than at cooler temperatures. These findings are similar to the relationship established for other myxozoans in their invertebrate hosts (El-Matbouli et al., 1999; Liyanage et al., 2003; Kerans et al., 2005; Tops et al., 2006; Tops et al., 2009; Xiao and Desser, 2000) and parallels the temperature dependent development of *C. shasta* in its fish host (Udey et al., 1975). As for *M. cerebralis* (El-Matbouli et al., 1999), temperature had an indirect effect on actinospore survival, with a greater

number of parasites persisting for a greater time at cooler temperatures however it was found that triactinomyxons may persist for as long as 15 days at 15ºC.

The development and release of *C. shasta* actinospores from *M. speciosa* is temperature dependent. Infected polychaetes acclimated to winter water temperatures (8ºC) released actinospores when transitioned to warmer temperatures (10 or 20ºC), but actinospores were not released from cohorts that remained at 4ºC over the 30 day experimental period. Peak actinospore release from *M. speciosa* acclimated to 20ºC occurred between days 11 and 15 and declined at days 18 and 30, whereas release at 10ºC also increased starting on day 11 but remained at approximately the same rate on days 18 and 30. Although we were unable to determine the length of parasite development at different temperatures because it was unknown when the polychaete became infected, this study demonstrates that *C. shasta* can "over-winter" in *M. speciosa,* and as water temperature increases in spring, parasites mature and actinospores are released. This explains the seasonality of fish infection which occurs at temperatures ≥ 7ºC (Ratliff, 1983; Hendrickson et al., 1989; Margolis et al., 1992). Additionally, we did not determine whether the warmer temperatures affected infection intensity in *M. speciosa* or merely maturation of the infection. Quantitative PCR and histology of the polychaetes would clarify this relationship.

Visual and molecular methods revealed a similar relationship between water temperature and actinospore release. Because of the small sample size analyzed by the visual method (only 0.5% of the volume that was analyzed by qPCR), comparing between the two methods cannot be done without being heavily biased. The low concentration of actinospores in the samples prevents the use of a hemacytometer and accurate estimates of parasite concentration. Additionally, the minimum detection threshold for the visual method is 200 actinospores/mL (based on seeing 1 actinospore in the 5µL examined), thus actinospores released in smaller quantities may have gone undetected. Molecular, DNA-based detection is much more sensitive but detects all stages of the parasite, even pre-mature stages that may have been released during the decay of a dead polychaete. This occurrence offers some explanation for the overlap of Cq values for 4 and 10ºC replicates as well as the detection of greater than one actinospore (based on Cq < 38) in samples for which

actinospores were not detected visually. Therefore the visual method verified the release of mature intact actinospores, but is less accurate quantitatively.

Viability of actinospores decreases with increasing temperature. *Ceratomyxa shasta* actinospores are fragile and full decomposition (separation of shell valves, nuclei and unfired polar capsules) occurred within minutes of extrusion of one of the polar capsules (personal observation, data not included). Although fish challenges were not conducted to confirm the visual observations, our laboratory studies corroborate the results of previous studies in which fish infection prevalence decreased when river water containing the parasite was held over time and at different temperatures (Ratliff, 1983; Foott et al., 2007).

In conclusion, temperature had no effect on the survival of *M. speciosa* infected with *C. shasta* but it did affect the progress of *C. shasta* infection and release of actinospores from this host and on the viability of the actinospore. Even though actinospores survived longer at cooler temperatures, the total actinospore release at 10ºC was still well below that at 20ºC. The shorter viability of actinospores at 20ºC may be offset by increased rate of development and maturity of new actinospores from the polychaete host. In the river, this could result in a smaller infective zone down stream of an infected *M. speciosa* population at warmer temperatures, but the actinospore dose in that zone is likely to be higher than it would be at cooler temperatures. Interpreting the affect of these influences on ceratomyxosis severity in fish in the river depends on a variety of factors such as the abundance and infection intensity of infected *M. speciosa* populations and the effects of prolonged exposure to low actinospore doses or very brief exposure to very high actinospore doses which are yet unknown.

Table 2.1. Average *Manayunkia speciosa* survival (%) ± S.D., and *Ceratomyxa shasta* infection prevalence (%)± S.D based on 3 replicates of 10 polychaetes each, except for 20ºC that only had 2 replicates. Survival and prevalence at the experimental temperatures are not significantly different (One way ANOVA p= 0.94 for survival, p=0.12 for prevalence).

	4°C	10°C	20°C
Survival (%)	70 ± 10	80 ± 17	70 ± 14
Prevalence (%)	100 ± 0	96 ± 6.4	83 ± 24

Table 2.2. Number of *Ceratomyxa shasta* actinospores visualized in a 5 µL aliquot over time at 4, 10 or 20ºC for each replicate. ND indicates no data due to the loss of this replicate.

	Days								
Temperature ºC	3	5	7	9	11	13	15	18	30
4	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0	0,0,0
10	0,0,0	0,0,0	0,0,0	0,0,0	0,0,6	0,1,0	0,0,0	2,0,1	0,0,2
20	0,0,0	0,0,0	0,0,0	0,0,0	6,1,ND	5,4,ND	8,2,ND	3,2,ND	2,1,ND

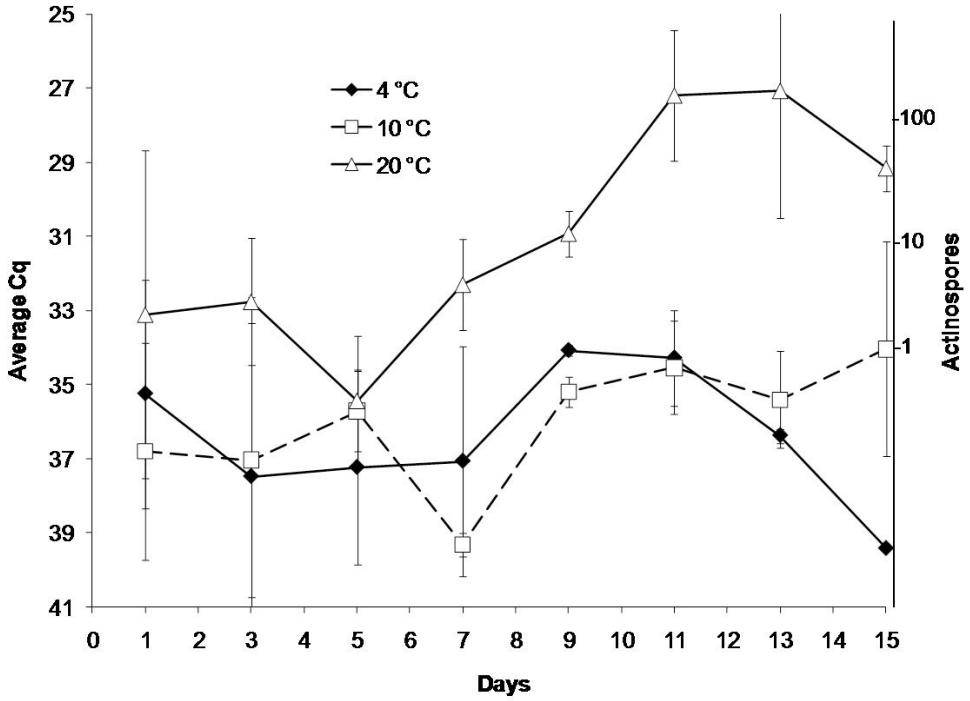

Figure 2.1. Relative abundance of *Ceratomyxa shasta* actinospores in the water after release from *Manayunkia speciosa* acclimated to 4, 10, or 20 ºC determine by qPCR of the water. Error bars indicate the standard deviation of the Cq values obtained from the three 1 mL replicate samples and the rate of actinospore release between temperatures is significantly different (Linear regression with interaction between temperature and time $p < 0.001$).

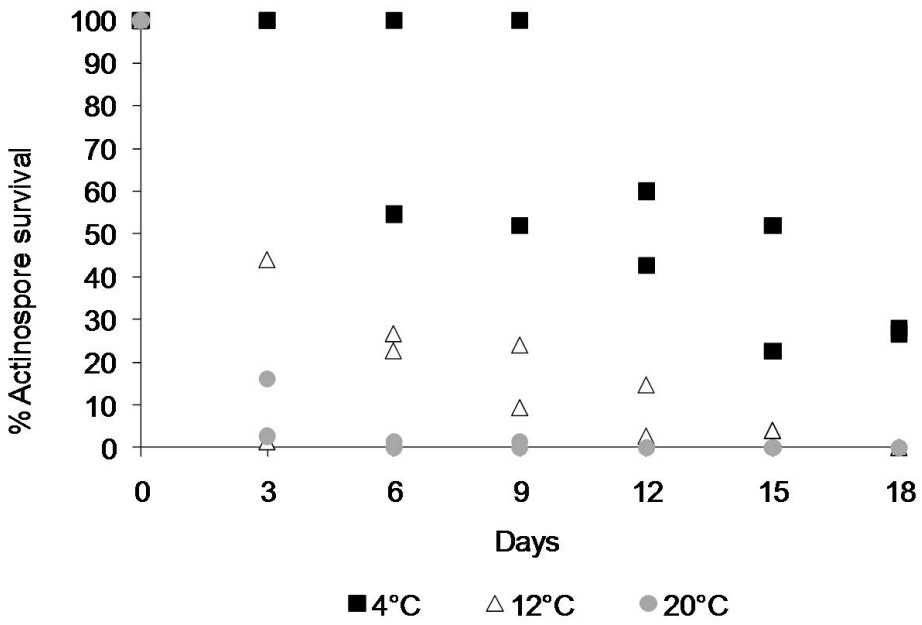

Figure 2.2. *Ceratomyxa shasta* actinospore viability over time from duplicate samples of actinospores held at 4, 12, or 20ºC.

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ACKNOWLEDGEMENTS

We express our sincere appreciation to Richard Stocking and Don Stevens who helped with polychaete collection as well as pump maintenance for the Willamette River water supply into the laboratory and the Nature Conservancy for access to our field site at Upper Klamath Lake and Dave Simon of Oregon State University Department of Fish and Wildlife for use their boat during sampling. We would also like to thank Sascha Hallett for review of this manuscript. This project was funded by the California Energy Commission Pulsed Flow Program and Oregon Sea Grant.

CHAPTER 3: *CERATOMYXA SHASTA* ACTINOSPORE RELEASE FROM THE POLYCHAETE HOST *MANAYUNKIA SPECIOSA*

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For submission to Journal of Parasitology as Research Notes

ABSTRACT

Ceratomyxa shasta, the causative agent of ceratomyxosis, is a myxosporean parasite of salmonids and has a complex lifecycle involving two hosts and two morphologically distinct spore stages: the actinospore and the myxospore. Actinospores are the parasite stage infectious to fish and develop in the dermis of *Manayunkia speciosa*, the freshwater polychaete host. It is hypothesized that actinospores are released through a secretory pore or directly through the dermis by osmotic pressure. In this study, live *M. speciosa* infected with *C. shasta* were monitored for release of actinospores. Packets of actinospores were observed exiting the worm through the dermis. Histology and transmission electron microscopy revealed a duct from the mucous cells where the actinospores mature, to the exterior of the worm (cuticle). Actinospore release does not appear to result in injury to the worm and timing of release appears continuous, correlating with actinospore maturity. Although actinospores appear to be released into the tube dwelling of the worm, movement of the worm in and out of the tube during feeding likely generates enough water circulation to transfer the spores into the water column.

Keywords: *Ceratomyxa shasta*, *Manayunkia speciosa*, polychaete, actinospore, release, mucus

INTRODUCTION

Manayunkia speciosa is a tube dwelling freshwater polychaete and is the definitive host of the salmon parasite *Ceratomyxa shasta* (Bartholomew et al., 1997). The actinospore of *C. shasta* develops in *M. speciosa* and, when released, infects salmon and trout and can cause the disease ceratomyxosis. In the fish, the parasite proliferates in the intestine causing hemorrhage, necrosis and death in severe infections. Myxospores that develop in the intestine of the fish host are released into the water column and are ingested by *M. speciosa*. In the polychaete, the parasite undergoes several pre-sporogonic divisions and migrates to the dermis where the actinospores mature (Meaders and Hendrickson, 2009).

In the original description of the parasite life cycle, Bartholomew et al. (1997) speculated that the actinospores were released through a secretory pore. Meaders and Hendrickson (2009) recently conducted a detailed examination of the infection in *M. speciosa* and document the course of the infection from ingestion of the myxospore to sporogony in the epidermis. The parasite penetrates the intestinal epithelium then migrates to the epidermis via the septal nerve. The authors propose that actinospores rupture the dermis and are released due to changes in osmotic pressure. However, their observations were made from histological sections of the worms, and the release of *C. shasta* actinospores from live *M. speciosa* is undocumented. The mode of spore release and health of the worm after release are important to our understanding of the pattern and timing of parasite release as well as the potential cumulative spore dose produced from one host. The goal of our study was to identify the mode of *C. shasta* actinospore release from *M. speciosa.*

MATERIALS AND METHODS

Infected polychaete colony: Sediment containing *M. speciosa* was collected from Upper Klamath Lake at the mouth of the Williamson River, OR in May 2007 (GPS: 42º 25.282’ N, 121º 55.836’ W). Material and lake water were transported in plastic bags supplied with oxygen to the John L. Fryer Salmon Disease Laboratory at Oregon State University, Corvallis, OR, USA. Colony maintenance and *C. shasta* infection proceeded as described by Bjork and Bartholomew (2009).

Actinospore release: Infected polychaetes were removed from the sediment and induced to vacate their tubes by gently tapping the tube with dental hooks. Only unoccupied portions of the tube were tapped to avoid damage to the polychaete. Live *M. speciosa* were placed on a microscope slide with approximately 50 µl of well water and allowed to move freely within the water droplet. An inverted microscope fitted with a Leica IM50 camera was used to capture images of the actinospores as they were released from *M. speciosa.* Observations were made over a 15 minute period.

The infected polychaete was then fixed for histology and actinospores released from the worms were fixed on slides with methanol and stained with 1% methyl green. *Manayunkia speciosa* were fixed in 10% neutral buffered formalin or in Davidson's fixative and processed by routine methods for histology at the Veterinary Diagnostic Laboratory at OSU. Histological sections were cut to 3 µm and stained with hematoxylin and eosin (H & E) or Alcian Blue (pH 2.5). Ten infected polychaetes and 12 uninfected polychaetes were examined.

To identify the host cell that the actinospores were released from, one infected *M. speciosa* was prepared for electron microscopy (Bartholomew et al., 1997). Briefly, the polychaete was fixed in 2% glutaraldehyde in phosphate buffered saline pH 7.4, for 2 hr at 4ºC then post fixed with 1% OsO_4 in the same buffer at room temperature. After dehydration, the polychaete was embedded in Spurr's embedding medium, then sectioned and stained with Reynold's lead citrate. Sections were examined and photographed with a Phillips CM12/STEM operated at 60 kV in transmission mode at the Electron Microscopy Laboratory, OSU.

RESULTS

Live observations: After vacating their tubes, *M. speciosa* were highly motile, moving around within the water droplet on the slide. Actinospores were observed being released from the body wall of live infected *M. speciosa* as the worm moved (Figure 3.1A-C). *Ceratomyxa shasta* development was asynchronous and only pansporocysts containing mature actinospores were observed exiting the worm. Pansporocysts consisted of 8 actinospores enclosed in a membrane which ruptured soon after release and the actinospores became free floating. A tube-shaped mucus

trail was left on the slide where *M. speciosa* traveled during the 15 min observation period and actinospores were also observed in this trail (image contrast was enhanced using Adobe Photoshop 7.0 to distinguish actinospores from mucus) (Figure 3.1D). The polychaete remained mobile and alive after actinospore release for several minutes prior to fixation, and any damage to the dermis was imperceptible.

Histology: Mature *C. shasta* actinospores and developmental stages were observed within the same polychaete (Figure 3.2). Pansporocysts developed in the dermis of *M. speciosa* surrounded by cells containing dense granules (Figure 3.2A). In uninfected worms, the dermis is composed of numerous granular mucous cells (data not shown). In the infected worm stained with Alcian Blue, the granule-containing mucous cells are less dense and are outnumbered by pink *C. shasta* infected cells (Figure 3.2A). In the H & E stained sections, the cuticle of *M. speciosa* is more distinct and an opening from a cell containing mature actinospores through the dermis and opening to outside of the cuticle was observed (Figure 3.2B).

Electron microscopy confirmed the observations of light microscopy: a duct leads from a cell containing *C. shasta* to the exterior of the worm (Figure 3.2C).

DISCUSSION

Observation of live *M. speciosa* releasing *C. shasta* actinospores corroborated observations by Meaders and Hendrickson (2009) that parasites are released directly through the dermis. However, actinospores appeared to be released through a duct in the dermis of the worm, rather than as a result of osmotic pressure as those authors suggest. Our observations add greater detail to this process and provide further support of the hypothesis of Bartholomew et al. (1997) that the actinospores are released from a mucous pore. Polychaete epidermis is comprised of 3 cell types: gland (secretory) cells, sensory cells and supportive cells (Hausen, 2005) and only secretory cells have a duct or pore to the exterior of the worm. In two sabellid polychaetes closely related to *M. speciosa*, *Timarete filigera* (Mastrodonato et al., 2006) and *Branchiomma luctuosum* (Mastrodonato et al., 2005), glandular cells excrete mucus to the surface of the worm through a duct. Histological comparison showed similar cell morphology between the mucus-secreting cells of these related species and the cells containing *C. shasta* actinospores in *M. speciosa*. Release of

actinospores did not result in rupture of the polychaete's dermis, providing further support for secretion through a duct or pore. In heavily infected *M. speciosa* late in infection, there is a reduction in the number of granule-containing (secretory) cells (Meaders and Hendrickson, 2009) in the dermis and remaining granules are squeezed between developing parasites. Thus, as *C. shasta* proliferates and matures, *M. speciosa* may have reduced mucus production which could lead to reduced host motility and tube construction based on the role of mucus in *Manayunkia aestuarina* (Lewis, 1968).

Ceratomyxa shasta is the first myxosporean for which parasite development and release has been documented to occur through the dermis of an annelid host. Other myxosporeans develop elsewhere in their invertebrate host such as the intestinal epithelium or coelom (El-Matbouli and Hoffmann, 1998; Hallett et al., 2001; Liyanage et al., 2003; Kallert et al., 2005; Szekely et al., 2005; Rangel et al., 2009). Parasites that develop in the intestinal epithelium are shed in the feces (El-Matbouli and Hoffmann, 1998). Of the species that develop in the coelom, one species migrated into the lumen of the intestine and was released through the feces (Hallett et al., 1998), whereas another species is presumably released at the time reproduction, however this observation coincided with the death of the host (Rangel et al., 2009). The use of the dermis indicates that *C. shasta* utilizes a unique strategy. The lack of evidence for pathogenic effects in the host during infection, and asynchronous development of actinospores indicate that *M. speciosa* can be heavily infected and release actinospores as they mature which is a common pattern for release of other myxosporeans from their respective annelid hosts (Yokoyama et al., 1993; El-Matbouli and Hoffmann, 1998a; Hallett et al., 1998).

Once actinospores are mature, and released from *M. speciosa*, they may still not be free in the water column. *Manayunkia speciosa* is a tube dwelling annelid and only its anterior end regularly extends from the tube for feeding, but actinospores develop in the posterior of the worm, in segments 6-10 (Meaders and Hendrickson, 2009). Thus, it is likely that actinospores are initially trapped in the mucus sheath that surrounds the body of *M. speciosa.* Mucus from the cell that the actinospore inhabits may still secrete some residual mucus, but mucus granules in infected cells are greatly

reduced. Therefore infected *M. speciosa* may have decreased mucus production in the posterior segments and actinospores would have fewer barriers in the access to free flowing water. For actinospores that are initially trapped in the mucus, we hypothesize that as the worm moves in and out of its tube for feeding, a current of water is produced which transports actinospores outside in the same manner that the host's excretory wastes are removed. Through asynchronous development and release via a mucus pore, *C. shasta* has evolved a relationship with *M. speciosa* in which multiple pansporocysts of actinospores can develop and be released with apparently minimal damage to the host.

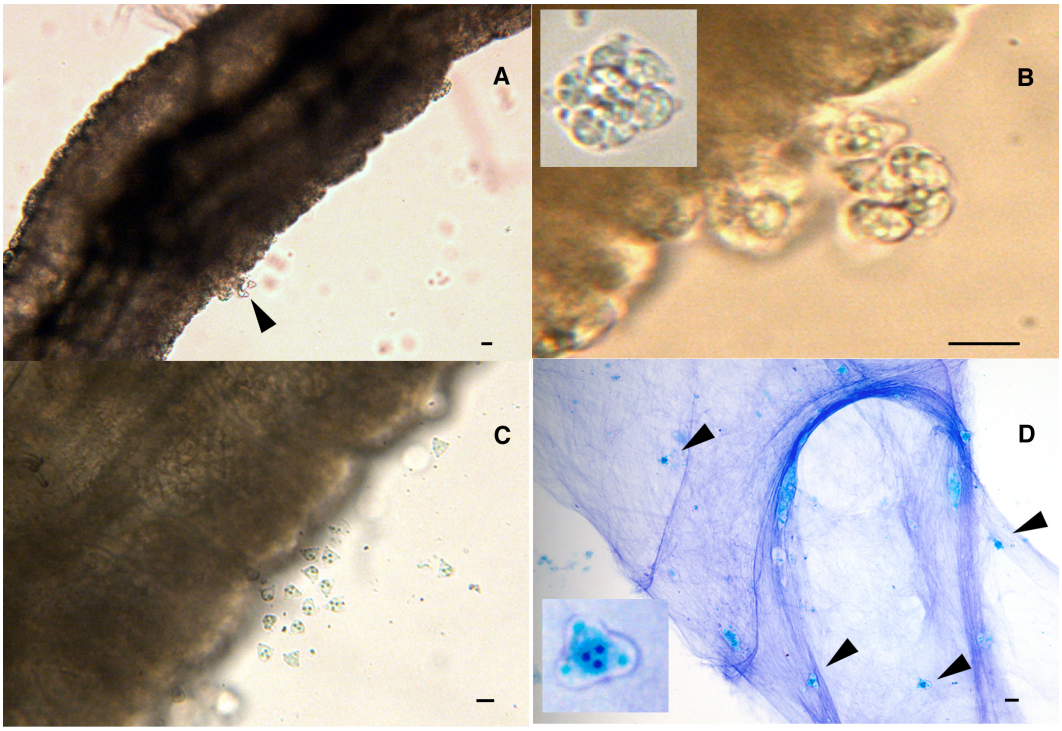

Figure 3.1. *Ceratomyxa shasta* actinospores secreted from *Manayunkia speciosa*. Viewed with light microscopy. Size bars are 10 µm. A. – C. Fresh, unstained material. A. Actinospores (arrow) secreted from *M. speciosa* dermis. B. Higher magnification of 2 pansporocysts exiting the polychaete dermis. Each pansporocyst is comprised of 8 actinospores (inset). C. Ruptured pansporocyst with free floating actinospores. D. Actinospores (arrows) trapped in the mucus trail of *M. speciosa*. Stained with Methyl green. Inset. Higher magnification of a single actinospore.

Figure 3.2. *Manayunkia speciosa* infected with *Ceratomyxa shasta*. Size bars are 10 µm. A.– B. Histological sections. C. Transmission electron micrograph. A. Alcian blue stain. Blue acidic mucus granules replaced by *C. shasta* pansporocysts. B. Hematoxylin and eosin stain. Actinospores in dermis with inset of higher magnification of actinospores in mucus pore. C. Host cell containing *C. shasta* (sp) with a duct (arrow) exiting to the cuticle (c).

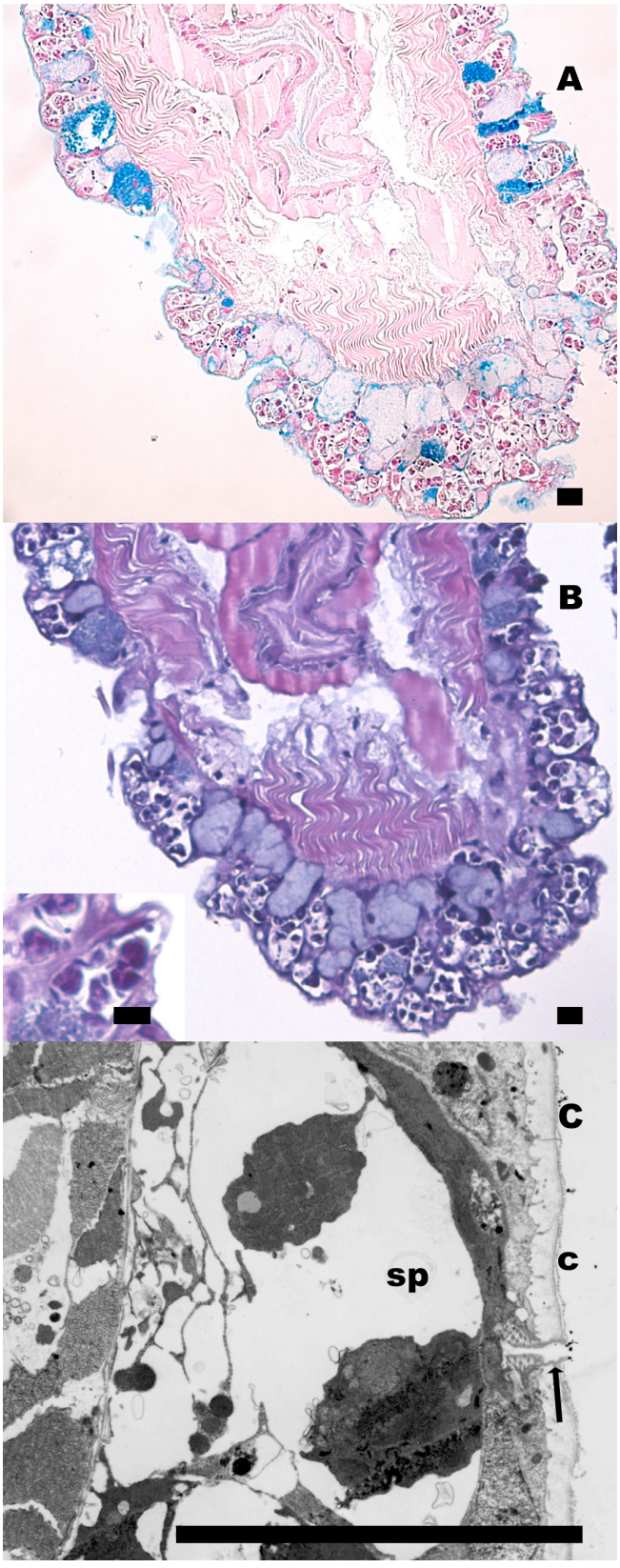

Figure 3.2

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ACKNOWLEDGEMENTS

We express our sincere appreciation to Richard Stocking and Don Stevens who helped with polychaete collection as well as pump maintenance for the Willamette River water supply into the laboratory and the Nature Conservancy for access to our field site at Upper Klamath Lake and Dave Simon and crew (Department of Fisheries and Wildlife, Oregon State University) for use of their boat for sampling. We also thank Dr. Sascha Hallett for review of this manuscript. This project was funded by the California Energy Commission Pulsed Flow Program and Oregon Sea Grant.

CHAPTER 4: THE EFECTS OF WATER VELOCITY ON THE *CERATOMYXA SHASTA* INFECTIOUS CYCLE

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Journal of Fish Diseases (2009) 32, 131-142

ABSTRACT

Ceratomyxa shasta is a myxozoan parasite identified as a contributor to salmon mortality in the Klamath River, USA. The parasite has a complex life cycle involving a freshwater polychaete, *Manayunkia speciosa* and a salmonid. As part of ongoing research on how environmental parameters influence parasite establishment and replication, we designed a laboratory experiment to examine the effect of water flow (velocity) on completion of the *C. shasta* infectious cycle. The experiment tested the effect of two water velocities 0.05 and 0.01 m/s, on survival and infection of *M. speciosa* as well as the transmission to susceptible rainbow trout (*Oncorhynchus mykiss*) and comparatively resistant Klamath River Chinook salmon (*Oncorhynchus tshawytscha*). The faster water velocity facilitated the greatest polychaete densities, but the lowest polychaete infection prevalence. Rainbow trout became infected in all treatments, but at the slower velocity had a shorter mean day to death, indicating a higher infectious dose. Infection was not detected in Chinook salmon even at a dose estimated to be as high as 80,000 actinospores per fish. The higher water velocity resulted in lower *C. shasta* infection prevalence in *M. speciosa* and decreased infection severity in fish. Another outcome of our experiment is the description of a system for maintaining and infecting *M. speciosa* in the laboratory.

Key words: *Ceratomyxa shasta*, infection, *Manayunkia speciosa*, parasite ecology, salmonids, water velocity

INTRODUCTION

The myxozoan *Ceratomyxa shasta* is enzootic among populations of salmon and trout in the larger river systems of the Pacific Northwest region of North America, including rivers in northern California (Ratliff, 1983; Ching and Munday, 1984; Hoffmaster et al., 1988; Hendrickson et al., 1989). In the Klamath River, which spans the California/Oregon border, *C. shasta* infection is associated with high mortality in juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum) (Foott et al., 2004) and there is great interest in finding ways to mitigate the impact of this parasite on these populations.

The life cycle of *C. shasta* requires two hosts: a salmonid host and *Manayunkia speciosa*, a freshwater polychaete (Bartholomew et al., 1997). In the salmonid, *C. shasta* infects the intestine causing hemorrhage and necrosis. Myxospore stages are shed by the fish in the late stages of infection (usually upon death of the fish) and infect the polychaete. Completion of the cycle occurs when actinospores are released from the polychaete and infect a fish.

In the fish host, *C. shasta* development is affected by temperature, the susceptibility of the fish and the infectious dose. Infection follows a seasonal trend (Hendrickson et al., 1989) and the rate of parasite development increases with increasing temperature (Udey et al., 1975). Fish stocks that originate or migrate through enzootic locations show the highest resistance (Bartholomew, 1998), but may succumb to infection as a result of prolonged exposure and presumably high actinospore doses (Ratliff, 1981). However, the threshold dose for infection in these stocks is unknown, and other than temperature, the environmental conditions that affect the progress of disease in fish have not been investigated.

Far less is known about interactions between *M. speciosa* and *C. shasta*, yet the parasite can only become established when the polychaete is present. Although stable substrate and moderate water velocities are described as two of the defining characteristics of *M. speciosa* habitat in the Klamath River Basin (Stocking and Bartholomew, 2007), little is known about factors affecting the colonization, reproduction, and lifespan of these worms (Croskery, 1978; Stocking and Bartholomew, 2007). The only reports of *C. shasta* infection in *M. speciosa* are from

Klamath River populations where infection prevalence ranged from < 1% to 8.3% (Stocking and Bartholomew, 2007). Reasons for the differences in infection prevalence are difficult to determine from field studies because of the inability to separate the effects of multiple variables such as temperature, flow, proximity to a myxospore source and myxospore dose. However, the relationship between high infection prevalence in polychaetes and severe infection in fish (Stocking and Bartholomew, 2007) indicates that we need to understand more about factors that facilitate parasite transmission between these hosts.

The relationship between water flow and parasite infection dynamics have been characterized for another myxozoan, *Myxobolus cerebralis*. In a laboratory study that examined the effects of flow on the transmission and ecology of *M. cerebralis*, Hallett and Bartholomew (2008) found a significant increase in parasite numbers and infection prevalence in the invertebrate host, *Tubifex tubifex*, under slower flow conditions. Parasite prevalence and infection severity in the fish host was also higher in the slow treatment groups. Similarly, an inverse relationship between water flow and *M. cerebralis* infection prevalence in fish was also demonstrated in a field study where water flows were much greater (E. R. Vincent, Montana Fish, Wildlife and Parks personal communication). These studies were the first to document the effects of water flow on a myxozoan life cycle, through changes in the number of invertebrate hosts as well as the infection prevalence and severity in both hosts.

We designed a laboratory model to test the hypothesis that water velocity affects completion of the *C. shasta* life cycle as well as *M. speciosa* survival. In our model, we tested the effects of two water velocities (0.05 and 0.01 m/s) on the survival and infection prevalence in *M. speciosa*, as well as the subsequent infection of susceptible and resistant fish hosts. Understanding how changes in water velocity affect the *C. shasta* infectious cycle will provide insight on temporal changes in infection, aid in predictions about fish infection severity based on river conditions, and could lead to control measures in rivers where flow can be manipulated.

MATERIALS AND METHODS

Collection of polychaetes: Polychaetes in sediment (sand and silt) were collected from the mouth of the Williamson River, a tributary of Klamath Lake in Oregon, USA on April 28, 2006. Samples were collected using an 83 µm mesh plankton net (17 cm diameter) fitted on a telescoping handle. Material was placed in plastic bags with approximately 500 mL of river water, supplied with oxygen and transported to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis, OR. Material was aerated overnight and the experiment was initiated the following day.

Experimental design: Four identical stainless steel tanks (~67 cm long) were divided into three replicate channels (each 10 cm wide) with plexiglass following the design of Hallett and Bartholomew (2008) (Figures 4.1 and 4.2). Water from the Willamette River (WR), Oregon, was supplied via a manifold behind the headwall to facilitate an even flow of water. The spill of water over the headwall created a plunge pool with turbulent flow in the upper one third of the channel. This hydraulic effect was decreased by the placement of a plexiglass plate fitted with twelve 0.95 cm holes drilled in a random pattern and placed 9.5 cm in front of the headwall. Polychaetes and their substrate (approximately 1.7 L) were randomly distributed in each of the replicate channels to a depth of approximately 3 cm, and allowed to settle 4 h prior to the initiation of water flow. The outflow of each channel was directed into individual 19 L aquaria to accommodate fish exposures.

Use of WR water insured an adequate food source for *M. speciosa*. However, because the WR supports the life cycle of *C. shasta*, we included two controls, one aquarium at each experimental velocity, to detect infection from the water source. Water was supplied at ambient river temperature for the duration of the experiment, which began April 29, 2006 and ended October 3, 2006. Temperature of the incoming WR water changed seasonally and ranged between 13.3 and 23.6 °C (USGS National Water Information System: Web Interface. http://waterdata.usgs.gov, Willamette River at Albany, OR site no. 14174000). Water temperature in each aquaria was measured during weeks 3, 9 and 14 and was within 2 °C of the mean reported temperature from the USGS monitoring station, regardless of fast or slow flow treatment.

Treatment groups: Water was supplied at 0.01 m/s to two of the tanks and one WR control creating the "slow" treatment groups. Water was supplied to the two remaining tanks and other WR control at 0.05 m/s for the "fast" treatment groups. The experimental slow velocity was selected based on the lowest measured velocity in the Klamath River where polychaetes were documented in this substrate (Stocking and Bartholomew, 2007). Although polychaetes were present in sand-silt at velocities as high as 0.15 m/s, the experimental fast flow was limited by the pump capacity (0.05 m/s). At initiation of the experiment, water velocity in the experimental channels was measured within 3 cm of the substrate level with a Marsh McBirney Flowmate 2000 (Frederick, MD) portable current velocity meter.

To test the effects of water velocity on *M. speciosa* infection, one tank of triplicate channels at each of the experimental velocities was seeded with *C. shasta* myxospores. To simulate natural myxospore introduction, a rainbow trout (*Oncorhynchus mykiss*, Walbaum) (average wt. 8.0 g) that had died as a result of severe ceratomyxosis was added to the head of each channel behind the eddy plate during week 3 as a source of myxospores, creating a "seeded" treatment. A non-infected rainbow trout was similarly added to the head of each of the channels of the other tanks at each velocity generating the "control" treatment. The four treatment groups will hereafter be identified as "slow control", "slow seeded", "fast control", and "fast seeded". WR controls will be identified as "WR fast" and "WR slow".

Myxospore estimation: To estimate the number of myxospores added to the system, the viscera and gills of similarly infected rainbow trout were removed from the fish. A modified centrifugation technique based on the procedure described by Bartholomew, Rohovec & Fryer (1989) was used to purify and quantify myxospores from each fish. Briefly, the tissues were mixed with 100 mL of well water and pulverized in a stomacher until they reached a slurry (about 5 min), to release myxospores. The solution was filtered through a 0.83 cm mesh screen to remove larger pieces of fish tissue, and rinsed with 125 mL water. This step was repeated using a 0.088 cm mesh screen. The total 350 mL spore solution was centrifuged at 1500 g, at 4°C for 20 min. Supernatant was removed and the pellet suspended in 200 mL water. This step was repeated and the final pellet suspended in 5 -10 mL of water.

Myxospore concentration was estimated by four separate counts on a hemacytometer, then multiplied by the volume to obtain the total myxospores from one fish. The estimates from four individual fish were averaged to provide the myxospore contribution from one fish in the seeded tanks.

Polychaete survival: Initial polychaete densities were determined from three 30 mL samples of sediment, randomly collected prior to the distribution of sediment to the tanks. To estimate the mean polychaete density of a channel, 30 mL sub-samples were randomly taken from three locations in each channel (below the inflow, in the middle and above the outflow) at weeks 6, 11, 15, and 22, and were fixed in 95% ethanol for polychaete density determination. The preserved substrate was emptied into a Petri dish in three 10 mL increments and under a dissecting microscope, polychaetes were separated from the substrate using modified dental tools. Polychaetes were counted at x65 magnification using a dissecting microscope. Higher magnification was used as necessary to confirm polychaete identification. Each channel was considered a replicate of the treatment group and densities from the three channels were averaged to estimate the polychaete density of that treatment group.

Polychaete infection: To determine whether water velocity had an effect on the infection prevalence in polychaete populations exposed to *C. shasta* myxospores during the experiment, the polychaetes collected for density determination in each treatment were assayed by polymerase chain reaction (PCR) in a pooled prevalence assay (Stocking and Bartholomew, 2007). In that study, the infection prevalence of *C. shasta* in *M. speciosa* from the Williamson River was estimated to be 0.45% (Stocking and Bartholomew, 2007). This estimate, and the estimated prevalence (0.7%) in *M. speciosa* at the time of collection for this experiment (week 0), was used to develop the pooling scheme for the non-seeded groups. Because of the addition of myxospores, the prevalence was expected to be higher in the seeded groups and the pooling scheme was adjusted to assay more individual worms (Williams and Moffitt, 2001). Table 1 illustrates the pool size, and number of pools used for comparison of infection prevalence between treatments and over time. The AusVet pooled prevalence calculator (Sergeant, 2004), which uses various pool sizes (number of *M.*

speciosa in a pool) and pool numbers (pools), was used to calculate the infection prevalence in *M. speciosa*.

Fish infection: To test the effect of water velocity on infection in susceptible fish, groups of either Trout Lodge (Trout Lodge, Sumner, WA) or Shasta strain (Sinnhuber Aquatic Research Laboratory, Oregon State University, Corvallis, OR) rainbow trout were exposed in aquaria under the outflow of each channel. Rainbow trout averaged 7.6 cm (± 0.4) and 5.9 g (± 1.3) at exposure. Groups of 10 fish were sequentially held in the aquaria for three separate exposure periods: weeks 1-6, weeks 6-10 and weeks 11-15. After exposure, surviving fish were held for 90 days in 12.8 ºC specific pathogen free (SPF) water. Aquaria were disinfected with Ido-Sept II (Mt. Hood Chemical, Portland, OR) between exposure groups.

To test the effect of the treatments on infection in a *C. shasta*-resistant fish strain, Klamath Chinook salmon (Iron Gate strain, Iron Gate Hatchery, CA, USA; average size 5.3 cm ± 0.4, 1.7 g ± 0.2) were added to the aquaria in place of rainbow trout at week 16. These fish were exposed only for one week because of high mortality caused by bacterial infections. The fish that survived this 1-week exposure were transferred to SPF water and replaced with a second group from the same cohort. This second group received a daily prophylactic treatment of TM-100 4% oxytetracycline medicated feed (Bio-Oregon, Longview, WA, USA) and was exposed for 4 weeks. Moribund fish were euthanized with tricaine methanesulfonate (MS222) (Argent Laboratories, Redmound, WA, USA).

Dead and moribund fish were examined for *C. shasta* myxospores in a wet mount of an intestinal scraping at x200 for 3 minutes (Bartholomew 2002). Intestinal tissue was collected from visually negative fish for assay by a *C. shasta*-specific polymerase chain reaction (PCR) as described by Palenzuela, Trobridge & Bartholomew (1999). Fish surviving 90 days were euthanized with MS222 and intestinal tissue samples removed and frozen until processed for PCR analysis. Fish infection prevalence was compared between treatment groups.

Statistical Analysis: All statistical analyses were performed using S Plus version 7.0 (Insightful Technologies, Seattle, WA, USA). Square root transformations were used to provide equal variance and meet the assumptions of the

statistical tests. ANOVA (with Bonferroni procedure when appropriate) tests were used to compare polychaete survival. ANOVA tests were also used to compare percent fish infection and mean day to death. Linear regression analysis was used to analyze polychaete infection prevalence trends over time.

RESULTS

Polychaete survival: Polychaete densities fluctuated throughout the course of the experiment (Figure 4.3). At week 0, there was a mean of 2837 *M. speciosa* per channel (SD = 567). The only significant difference between polychaete densities at any sampling time was between the fast and slow control groups at week 15. The average polychaete density declined to less than 1500 polychaetes per channel in all treatments at week 6. Although there was a trend towards lower polychaete densities in the fast groups at this time, they were not significantly different from the slow groups. By week 10, densities in all treatments had increased, but densities in the fast channels exceeded those of the slower channels, with the fast control treatment having the highest mean density of 3465 (SD = 1574) polychaetes per channel, followed by the fast seeded treatment with a mean density of 2664 (SD = 1503). The mean density of the slow seeded group was 1987 (SD = 596) per channel at week 10, and the slow control channels had a mean density of 1297 (SD = 978). The densities of all groups, except for fast control, peaked at week 10 then declined. Densities in the fast control treatment group continued to increase to a mean 5948 (SD = 3052) polychaetes per channel at week 15 then declined. At week 22, the mean density was less than 1000 polychaetes per channel for all of the treatment groups. As a result of high variability between sub-samples and channels, only the trends in fast control and slow control are significantly different from each other, with the largest difference occurring at week 15 ($P = 0.017$ linear regression). As the experiment progressed, there was a seasonal warming of river temperature that peaked (23 °C) between weeks 8 and 22 (Figure 4.4). However, there were no observed differences in water temperature of the tanks at fast and slow flow.

Myxospore estimate: The average myxospore estimate was 4.9×10^7 myxospores per fish (range 5.2×10^5 - 1.1×10^8).

Polychaete infection: The estimated infection prevalence among polychaetes at week 0 was 0.7% (Figure 4.5). Myxospores were added only to the seeded treatments, therefore the infection prevalence in the control channels throughout the experiment, and at week 0 for all treatment groups, is considered to be the background infection prevalence and was estimated to be as high as 3.86%. At week 6, there was not a significant difference in infection between any treatments, although the slow seeded group had a higher prevalence than the fast seeded treatment (Figure 4.5, Table 4.1). Infection in the fast and slow seeded treatments at week 10 was 2.8 ± 2.6 % (SD) and 2.7 ± 2.4 % (SD), respectively, with no significant difference between either group or the controls. At week 15, mean infection prevalence in the slow seeded group reached 14 ± 8.4 % (SD), whereas mean prevalence in all other treatment groups was below 2%. This peak in the slow seeded group is the only significantly different observation among all of the sample periods ($P = 0.009$ ANOVA after arcsine transformation). None of the polychaetes assayed at week 22 were positive for *C. shasta* infection.

Fish infection: Susceptible rainbow trout became infected during all exposures and mortality was high in all exposure groups and exposure periods (Figure 4.6). Because of fish loss through escape (through the standpipe or tank lid), the number of fish assayed by PCR did not equal ten for all groups. To ensure that exposure time was equal for the groups compared, only groups that had at least five living fish at the time of the first *C. shasta* mortality for that exposure period were included in the analysis; one replicate of the fast seeded treatment was excluded during the first and third exposure periods.

All susceptible rainbow trout exposed during the first 6 weeks had fatal *C. shasta* infections, except for those in the WR groups. Only one fish held in the WR control slow tank during this period was positive by PCR but the infection was not fatal. The infection prevalence in this group (10%) was significantly lower than all the treatment groups ($p<0.001$ one way ANOVA with Bonferroni procedure on arcsine transformed data).

In weeks 6-10, *C. shasta* associated mortality in rainbow trout exposed in the fast seeded treatment (mean 82%) was significantly less than in fish exposed in the

other treatment groups during the same time (99.6 to 100%) ($p= 0.014$ from ANOVA after arcsine transformation). Mortality between groups exposed during weeks 11 to 15 was not significantly different ($p= 0.09$). No WR exposed fish became infected after the first 6 week exposure. Only fish from the same exposure period were compared because of the variation in temperature between exposure groups.

The mean day to death of susceptible fish exposed in the slow treatments was significantly lower than those exposed to the fast velocity during all exposure periods ($p<0.001$) (Figure 4.6). In the first exposure period, fish exposed in the slow treatment groups died an average of 11 days earlier than the fast exposure groups. For the second and third exposure periods, this difference decreased to 3 and 4 days, respectively. Differences between mean day to death of the seeded and control treatments at either velocity were not significantly different.

No Chinook salmon succumbed to *C. shasta* infection during either a 1 week or 4 week exposure or assayed positive by PCR.

DISCUSSION

Environmental conditions are inextricably linked to a multitude of processes vital to the *C. shasta* host parasite relationship. Water velocity can affect water temperature, which affects the rate of myxospore development in the fish host (Udey et al., 1975). Thus, it has the potential to affect parasite concentration, extent of distribution, and residence time of the parasite in an area. Although the relationship between water velocity and salmon migration rate is complex, multiple studies report an association between decreased water velocity or water flow and increased migration times (Smith et al., 2002; Plumb et al., 2006). Increased migration times likely increase the exposure time of fish to the parasite. In this laboratory study, we investigated the effects of water velocity on the invertebrate host population density and *C. shasta* infection prevalence in the invertebrate and fish hosts.

In the experimental channels, flow had a significant effect on polychaete density, with higher mean polychaete densities occurring in the fast treatment groups at 0.05 m/s than at the slow rate of 0.01 m/s. Although *M. speciosa* did survive at the slow flow, peak densities were not as high except at week six. At this time, all of the

populations appear to have been experiencing a decline, perhaps as a result of acclimation to experimental conditions or handling stress after collection from the river. Thereafter, the fast flow treatment groups had higher mean polychaete densities at all time points, indicating that reproduction was occurring in these groups.

Higher *M. speciosa* densities at the faster flow is consistent with the peak densities found in sand-fine benthic organic matter at 0.05m/s in the Klamath (Stocking and Bartholomew, 2007) and Ottawa rivers (Mackie and Qadri, 1971). The fast flow (0.05 m/s) represents a modest flow when considering the range at which *M. speciosa* is reported in this substrate (0.01 to 0.15 m/s). The ability of *M. speciosa* to colonize other habitats (i.e. *Cladophora* sp.) appears to facilitate their ability to survive higher velocities and to persist after high flow events (Stocking and Bartholomew, 2007). The higher population density of *M. speciosa* at the fast velocity in this experiment suggests that polychaetes require sufficient flow to transport nutrients and carry away wastes without disturbing the microhabitat.

Polychaete infection prevalence was affected by water velocity, with prevalence significantly higher (average prevalence 14.4%) in the slow seeded treatment group, 12 weeks after the addition of myxospores. This finding parallels that of Hallett and Bartholomew (2008), who found a higher infection prevalence of *M. cerebralis* in *T. tubifex* when exposed to an infected fish in a slow flow compared to a ten-fold faster flow. In our study, variation in infection prevalence within treatment replicates was high, but this may be a reflection of the natural variation in the rate of myxospore dispersal from a dead fish. Although the infectious dose for a polychaete is unknown, at least 180 myxospores were available per polychaete and this resulted in a significant increase in infection prevalence under slow flow conditions.

Polychaete densities were not significantly different between the seeded and control groups of the same water velocities, indicating that infection had little effect on survival. However, the inability to monitor individual polychaetes during the course of an infection makes it difficult to interpret the effect of *C. shasta* infection

on polychaete survival. Studies utilizing higher polychaete infection prevalence and more frequent monitoring may yield more conclusive results.

Infection in susceptible fish exposed over the course of the study was high at either velocity and at all levels of polychaete infection prevalence. The natural background infection prevalence in the *M. speciosa* collected from the Williamson River (3.86 % or lower) was sufficient to cause mortal infections in susceptible rainbow trout. At week 0, this equates to as many as 110 infected polychaetes in each channel. One infected polychaete has the potential to produce several thousand actinospores (authors' personal observation), therefore even the low number of infected polychaetes in the control (non-seeded) treatments was capable of producing sufficient actinospores to overcome the low resistance of the rainbow trout. The overall high mortality (except in the controls) and low variability within treatment groups made it unlikely that exclusion of one replicate (based on low fish numbers) in the first and third exposure periods biased the analysis.

No Chinook salmon became infected by *C. shasta* during either a 1 week or 4 week exposure. Based on the polychaete density and infection prevalence at the time of exposure, it is estimated that an average of 207 infected polychaetes per channel were present in the slow seeded treatment group. The number of actinospores released from an infected polychaete will vary based on the degree of infection, however, we have observed over 2,000 mature actinospores in a single heavily infected polychaete (author's unpublished data). When this estimate is applied to the average number of infected polychaetes, the group of Chinook salmon had a cumulative exposure of greater than 410,000 actinospores. Although this is a gross estimate, it indicates that this stock may be resistant to as high as 82,000 actinospores per fish. This level of resistance is supported by a field study in which 37% of Trinity River Chinook salmon did become infected when exposed to at least 300,000 actinospores (J. S. Foott, personal communication). Controlled studies utilizing higher actinospore concentrations are needed to determine the infectious dose for Chinook salmon and characterize the conditions (such as water velocity and temperature) that facilitate infection.

Although there was no difference in the number of fish infected, the mean day to death was lower for rainbow trout exposed in the slow velocity treatments, indicating these fish received a higher infectious dose. In a study conducted in a natural system, E. R. Vincent, Montana Fish, Wildlife and Parks (personal communication) demonstrated an increase in intensity of parasite infection in fish at slow flows. In that study, the inverse relationship between flow and intensity of infection was attributed to a dilution effect of the infectious agent in large volumes of water, as would occur at high flow rates. Contrary to this conclusion, another *C. shasta* study reported that a three fold increase in water flow did not coincide with either a reduction in parasite DNA detection in water samples or fish infection prevalence (Foott et al., 2007). Hallett and Bartholomew (2008) also found evidence of increased *M. cerebralis* actinospore and fish interactions at a slower water flow. These data indicate that factors other than dilution, such as the ability of the parasite to find and interact with the fish host, may be affected by water velocity.

One additional outcome of our research was the establishment of a laboratory culture system for *M. speciosa.* Research on *M. cerebralis* progressed rapidly once populations of the alternate hosts could be sustained and the life cycle completed in the laboratory (Wolf and Markiw, 1983). This was accomplished with some degree of success in this study. The difficulties in maintaining *M. speciosa* led to pilot tests of different food and water sources, with stable populations established in sand-silt through the use of WR water as a source of nutrients. Under these conditions, polychaete populations were maintained in the laboratory for up to six months and in the fast control treatment, population densities increased significantly.

Although this study was successful in completing the *C. shasta* life cycle, there were limitations in the experimental design. First, the relationships determined in this study are limited to *M. speciosa* populations in sand-silt and at relatively slow velocities when compared with the range occurring in real river systems. We limited our studies to this habitat because of the poor survival of *M. speciosa* in *Cladophora* sp. in pilot studies, probably as a result of damage to the worm when this material is scraped from rocks and boulders. Given the occurrence of *M. speciosa* populations in *Cladophora* sp. at velocities greater than 0.2 m/s (Stocking and Bartholomew, 2007),

the trend toward higher population densities at higher velocities as demonstrated in this experiment may be even more distinct in this substrate. Second, the experimental velocities were limited by the pump capacity, and both are relatively slow compared to velocities in which *M. speciosa* populations are reported. Third, the culture system relies on polychaete collection from wild populations, which often have a background level of *C. shasta* infection. This background level was responsible for causing lethal *C. shasta* infections in susceptible fish even when the polychaete populations were not seeded with myxospores. This natural infection prevalence prevented us from distinguishing the effect of the two velocities on fish infection prevalence.

The open design of the system was also vulnerable to changes in the WR. It is unclear if factors such as temperature and sediment load influenced the survival of *M. speciosa* or the rate of actinospore production. Although the use of WR water introduced the risk of adding myxospores and actinospores to the system, there was no significant increase in *M. speciosa* infection prevalence or fish infection prevalence in any of the controls indicating that this risk was not realized. In spite of these limitations, this system maintained polychaetes at relatively high infection levels and offers many opportunities to answer questions about the *C. shasta* life cycle. For example, the peak in polychaete infection prevalence 12 weeks after the addition of an infected fish provides some indication of the amount of time it takes for myxospores to be released from a dead fish and infection in *M. speciosa* to develop.

The goal of this study was to determine the effect of water velocity on *M. speciosa* populations and the *C. shasta* infectious cycle. The faster water velocity was associated with decreased *M. speciosa* infection prevalence and longer survival of infected susceptible fish. At the slower velocity, *M. speciosa* had higher infection prevalence and poorer survival. As a means to control *C. shasta,* water velocity manipulation has the potential to alter *M. speciosa* population densities, *M. speciosa* infection prevalence and fish infection severity. Water flow manipulations may influence fish residence and migration times, thus, further affecting the likelihood of infection. The velocities tested in this experiment were limited by the capacity of the system, allowing a comparison of only a five-fold difference in velocity. Therefore,

the differences in infection and survival demonstrated in this experiment may be even more distinct at higher water velocities in the wild.

Table 4.1. Summary of samples used to estimate the apparent prevalence of *Ceratomyxa shasta* in *Manayunkia speciosa* collected from each treatment group throughout the experiment

Treatment	Week	Pool size and number of pools tested (*n*)	Pools positive	Total polychaetes	% Infection
None	0	1 (10), 5 (10), 8 (1), 10 (8)	0,0,0,1	161	0.7
Fast seeded	6				
1		1 (10), 4 (1)	0,0	14	0
2		1 (10), 4 (1), 5 (1)	0,0,0	19	0
3		1 (10), 4 (1), 5 (3)	0,0,0	29	0
Fast control	6				
1		5 (3), 6 (1)	0,0	21	0
2		1 (2), 5 (6)	0,0	31	0
3		3 (1), 5 (5)	0,1	28	3.86
Slow seeded	6				
1		1 (10), 5 (11), 10 (2)	0,1,0	75	1.21
2		1 (10), 4 (1), 5 (6)	0,0,1	44	2.38
3		1 (10), 5 (10), 10 (2)	0,0,0	80	0
Slow control	6				
1		5 (8)	1	40	2.64
2		2 (1), 5 (11), 10 (4)	0,2,0	97	2.15
3		3 (1), 5 (6)	0,1	33	3.23
Fast seeded	10				
1		1 (10), 4 (1), 5 (7)	0,0,1	49	2.13
2		1 (10), 3 (1), 5 (10), 10 (10), 20 (1)	0,1,0,0,0	193	0.55
3		1 (10), 5 (11), 10 (11)	0,2,6	175	5.7
Fast control	10				
1		5 (10), 10 (11), 20 (2)	0,2,0	180	1.05
2		3 (1), 5 (10), 10 (1)	0,0,0	63	0
3		5 (10), 8 (1), 10 (9), 20 (5)	0,0,0,0	248	0
Slow seeded	10				
1		1 (10), 3 (1), 5 (10), 10 (5)	0,0,4,0	113	3.82
2		1 (10), 5 (10), 9 (1), 10 (7)	0,3,0,2	139	4.39
3		1 (10), 5 (11)	0,0	65	0
Slow control	10				
1		1 (1), 5 (2)	0,0	11	0
2		2 (1), 5 (10), 10 (3)	0,0,0	82	0
3		5 (10), 10 (6)	0,0	110	0
Fast seeded	15				
1		1 (10), 4 (1), 5 (11)	0,0,0	69	0
2		1 (10), 5 (3)	0,0	25	0
3		1 (10), 5 (20), 10 (5)	0,2,0	160	3.42
Fast control	15				
1		5 (11), 10 (10), 20 (5)	0,1,0	255	0.4
2		3 (1), 5 (10), 10 (9)	0,1,0	143	0.7
3		5 (10), 10 (10), 20 (5)	0,1,1	250	0.9
Slow seeded	15				
1		1 (10), 3 (1), 5 (22), 10 (5)	0,1,5,2	173	5.28
2		1 (20), 3 (1), 5 (1)	2,1,1	28	16.3
3		1 (21), 2 (1)	5,0	23	21.74
Slow control	15				
1		4 (1), 5 (5)	0,0	29	0
2		5 (4)	0	20	0
3		5 (3)	0	15	0
Fast seeded	22				
1		1 (1)	0	1	0
2		1 (10), 3 (1), 5 (9), 10 (1)	0,0,0,0	68	0
3		1 (2)	0	2	0
Fast control	22				
1		2 (1), 10 (3)	0	32	0
2		10 (7)	0	70	0
3		10 (4)	0	40	0
Slow seeded	22				
1		1 (4)	0	4	0
2		1 (8)	0	8	0
3		1 (10)	0	10	0
Slow control	22				
1		2 (1)	0	2	0
2		4 (1)	0	4	0
3		0	0	0	0

The number of pools, pool size (polychaetes in each pool), positive pools, total polychaetes assayed per sub-sample and percent infection obtained from the AusVet pooled prevalence calculator are listed.

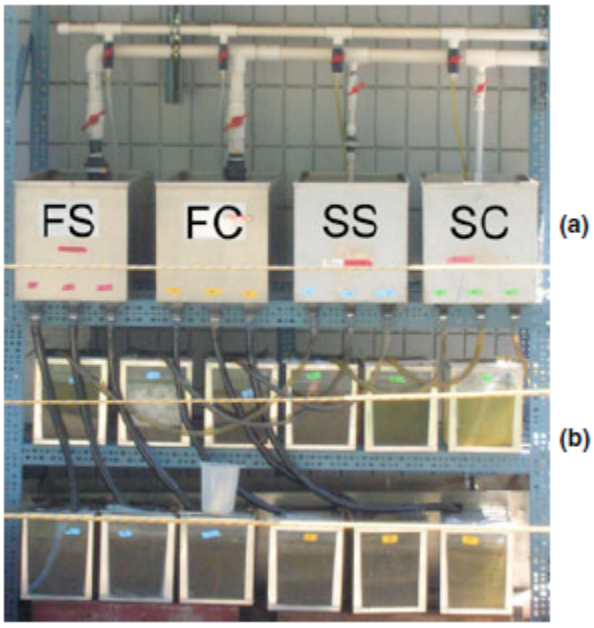

Figure 4.1. Stainless steel tanks used to test fast and slow flow conditions. a) Experimental flow tanks divided into replicate channels of the treatment groups [fast seeded (FS), fast control (FC), slow seeded (SS) and slow control (SC)] containing sediment and polychaetes. b) Aquaria in the outflow of the polychaete tanks for fish exposures.

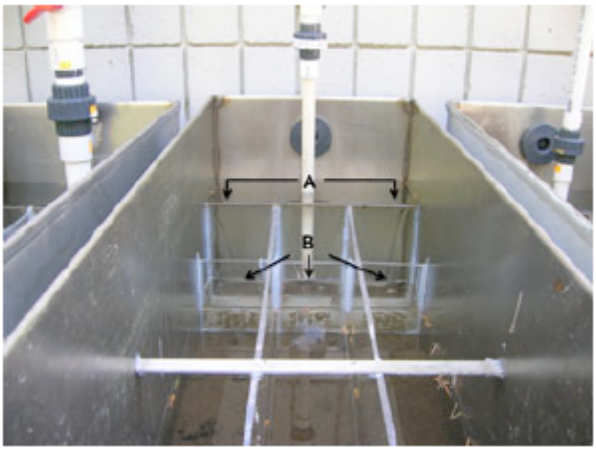

Figure 4.2. A treatment tank with replicate channels. The common inflow water is split and flows into a header section of each tank, then spills equally over a plexiglass divider (A) into the three replicate channels (B).

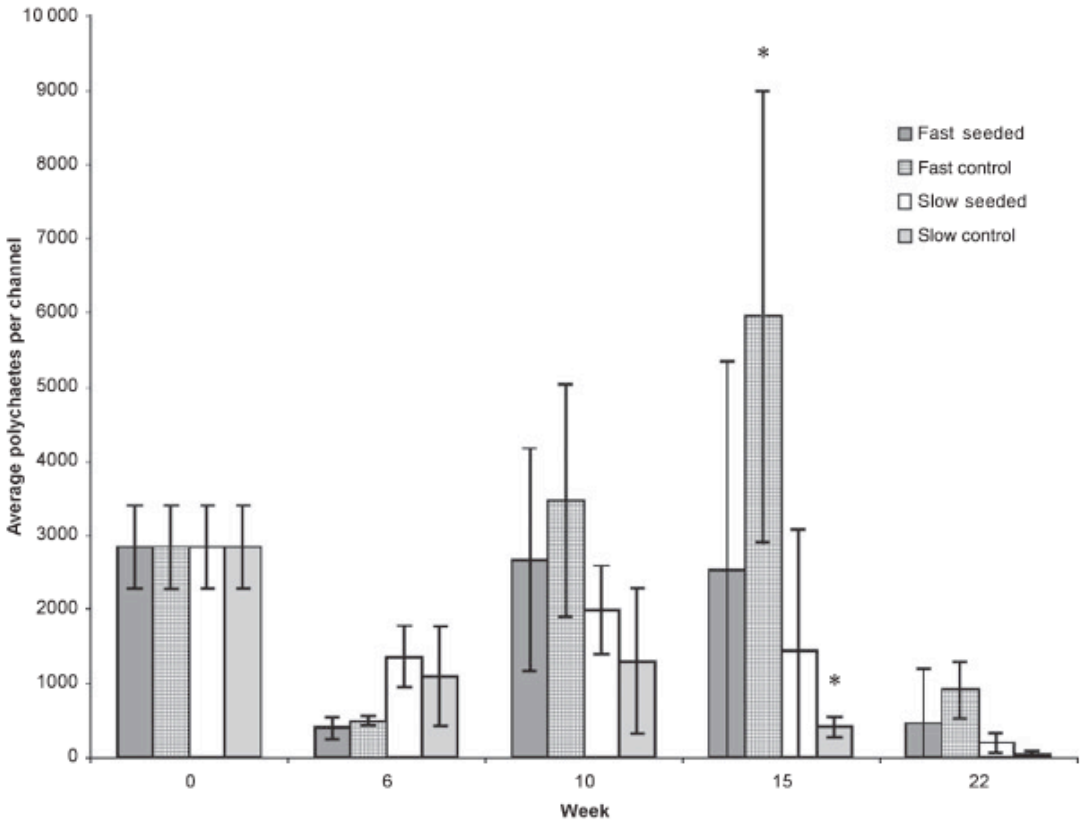

Figure 4.3. *Manayunkia speciosa* densities at slow (0.01 m/s) and fast (0.05 m/s) water velocities, with and without addition of *Ceratomyxa shasta* myxospores. Error bars indicate standard deviation. Asterisks indicate observations that are significantly different.

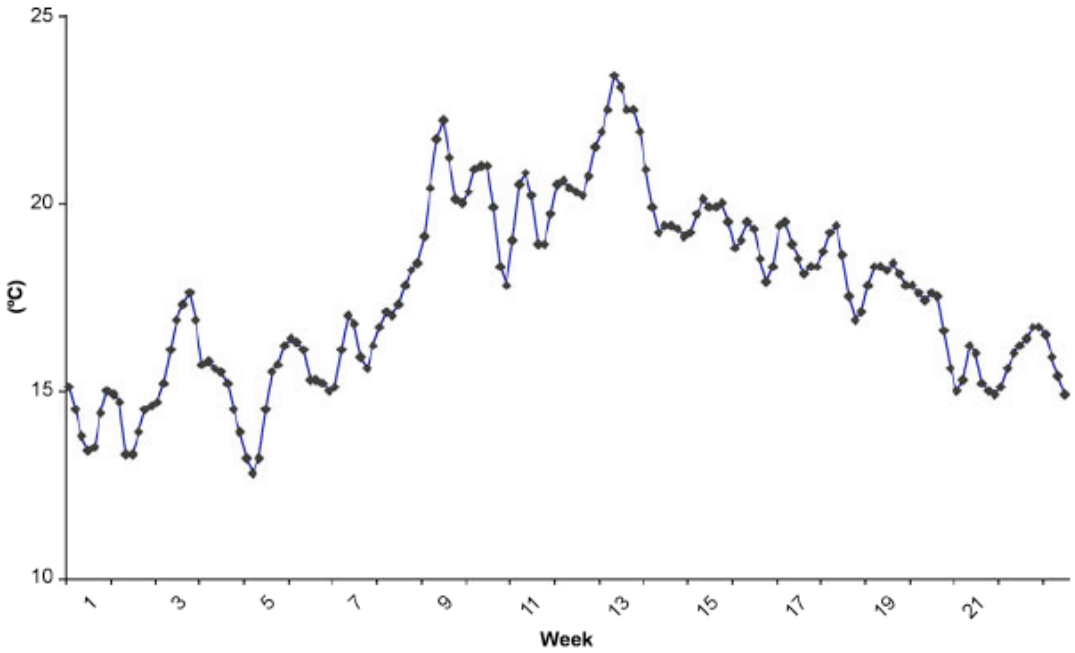

Figure 4.4. Willamette River temperature during the experiment obtained from USGS Daily Surface Water at Albany, Oregon (14174000).

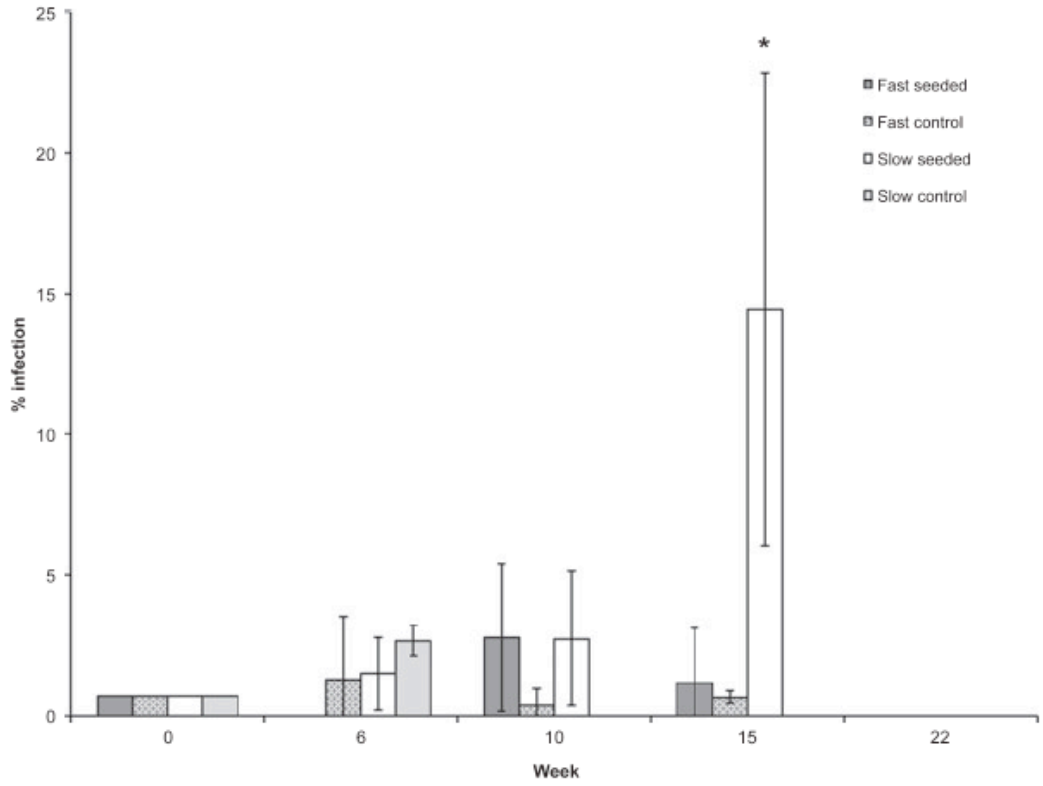

Figure 4.5. *Manayunkia speciosa* infection prevalence at slow (0.01 m/s) and fast (0.05 m/s) water velocities, with and without the addition of myxospores at three weeks. Error bars indicate standard deviation. Asterisks indicate observations that are significantly different.

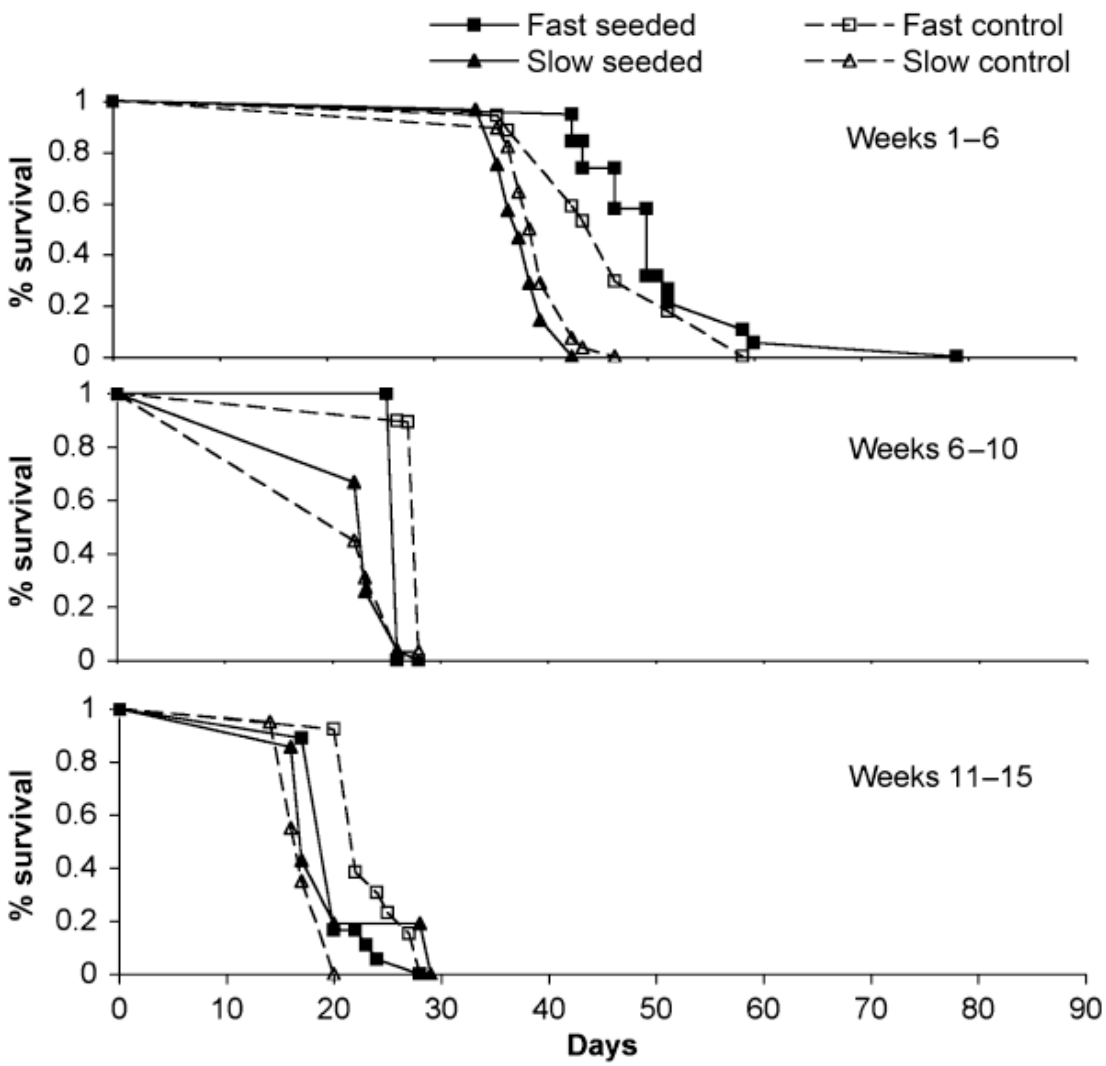

Figure 4.6. Survival curves for susceptible rainbow trout from the three exposures groups. No *Ceratomyxa shasta* associated mortality occurred in the Willamette River groups in any exposure period.

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ACKNOWLEDGEMENTS

The authors sincerely thank Don Stevens, of the John L. Fryer Salmon Disease Laboratory at Oregon State University, for the construction and plumbing necessary to conduct this experiment, in addition to his help collecting material from the river. We thank the Nature Conservancy for access to the Williamson River sampling site and Dave Simon and crew (Department of Fisheries and Wildlife, Oregon State University) for use of their boat for sampling. Also from Oregon State University, we acknowledge Sascha Hallett for her guidance in experimental set up and manuscript preparation; Harriet Lorz for assisting in the maintenance of fish in the experiments; Carly Todd who helped sort polychaetes for density determination; Lindsey Osborn for assistance with pilot studies; Jack Giovanini and Doug Larmour for their assistance in statistical analysis. The Sinnhuber Aquatic Research Laboratory, Oregon State University, supplied Shasta strain rainbow trout through NIH.NIEHS grant number ES00210. This study was funded by the Public Interest Energy Research Program of California Energy Commission through the Pulsed Flow Program of the Center of Aquatic Biology and Aquaculture of the University of California, Davis. Additional funding was provided by the Fish and Wildlife Service through the Klamath River Basin Fisheries Task Force.

CHAPTER 5: EFFECTS OF *CERATOMYXA SHASTA* DOSE ON A SUSCEPTIBLE STRAIN OF RAINBOW TROUT AND COMPARATIVELY RESISTANT CHINOOK AND COHO SALMON

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Diseases of Aquatic Organisms (2009) 86, 29-37

ABSTRACT

Ceratomyxa shasta infects salmon and trout, causing ceratomyxosis, a disease characterized by parasite proliferation in the intestine and death. We used laboratory challenges to investigate the infective dose for three fish species: a susceptible strain of rainbow trout *Oncorhynchus mykiss* and comparatively resistant Chinook *O. tshawytscha* and coho salmon *O. kisutch.* For susceptible rainbow trout, we determined the outcome of infection under conditions of varying parasite dose, fish size, and parasite concentration. A single actinospore was sufficient to cause a lethal infection in susceptible rainbow trout. The mean days to death (MDD) did not significantly decrease among doses causing 100% prevalence, indicating a minimum time required for parasites to replicate to a fatal level. When dose was constant, but delivered in a higher parasite concentration, higher infection prevalence and mortality resulted. One actinospore per fish caused 57% infection and mortality in fish challenged in 0.5 L of water, whereas 10 spores per fish resulted in an average of 49% infection and mortality in 1 L challenges. This effect is most likely due to a higher encounter rate in the smaller water volume. Neither infection prevalence nor MDD was significantly different between large trout (84.9 g) and small trout (6.3 g). Chinook salmon did not become infected even when challenged with 5,000 actinospores. One fatal infection occurred in coho salmon challenged with 1,000 actinospores. This study confirms that even low doses of *C. shasta* cause severe infection in highly susceptible fish, describes the dose response on MDD, and demonstrates that parasite concentration influences infection prevalence.

Keywords: *Ceratomyxa shasta*, infective dose, rainbow trout, *Manayunkia speciosa*, fish size, Chinook salmon, coho salmon

INTRODUCTION

The myxozoan parasite *Ceratomyxa shasta* causes ceratomyxosis in salmonid fishes, a disease characterized by hemorrhage and necrosis of the intestine. The parasite is endemic in certain rivers of the Pacific Northwest of the United States and Canada, and infections are reported from many species of wild and hatchery-reared salmonids (Sanders et al., 1970; Margolis and Evelyn, 1975; Zinn et al., 1977; Ratliff, 1981; Ching and Munday, 1984). The severity of infection depends on a variety of factors such as water temperature (Udey et al., 1975), the inherent resistance of the fish host (Zinn et al., 1977; Ching and Munday, 1984; Hemmingsen et al., 1986; Ibarra et al., 1992b; Bartholomew, 1998; Bartholomew et al., 2001), and parasite dose (Ratliff, 1983). The infective dose (number or parasites required to infect the host), the lethal dose (number of parasites required to kill the host) and factors influencing susceptibility (such as fish size) are not thoroughly understood.

Until recently, infecting fish with *C. shasta* relied on natural challenge (exposure) to the parasite. Studies using this method demonstrated a wide range in *C. shasta* susceptibility between salmonid species and strains but did not allow determination of parasite dose during the exposure. Zinn et al. (1977) determined that numerous salmon and trout species are susceptible to the parasite; however, susceptibility within a species is highly variable. Strains from endemic areas, such as the Fraser, Columbia and Klamath River Basins, were shown to have higher resistance to the parasite (reduced infection prevalence and mortality) than strains from areas free of the parasite (Zinn et al., 1977; Buchanan et al., 1983; Hendrickson et al., 1989; Bartholomew, 1998). However, even strains that demonstrate resistance became infected and died when the parasite challenge was sufficiently high (Ratliff, 1981; Ibarra et al., 1992b; Bartholomew et al., 2004).

Despite the limitations of using natural exposures, Ratliff (1983) estimated that one *C. shasta* was capable of causing fatal infection in susceptible rainbow trout, *Oncorhynchus mykiss* by serially diluting infectious water until mortality no longer occurred. Similarly, Johnson (1975) and Ibarra et al. (1992a) transmitted *C. shasta* to fish through intraperitoneal injection of as few as one *C. shasta* trophozoite (developmental stage in the fish). Interpretation of these studies is limited by the

inability to quantify the parasite in the former and by the artificial route of infection in the latter. However, they indicate that in susceptible strains of fish, *C. shasta* is highly pathogenic and only a small infective dose, as low as one parasite, causes fatal infection.

Controlled challenges became possible with the establishment of the parasite's two-host life cycle (Bartholomew et al., 1997) in the laboratory (Bjork and Bartholomew (2009a). Infection of *Manayunkia speciosa,* the freshwater polychaete host of *C. shasta* with myxospores from infected fish results in production of actinospores. The actinospores can be harvested from infected polychaetes for direct fish challenges.

Here we investigated the infective dose for susceptible rainbow trout, the relationship between parasite concentration and infective dose, the effects of fish size on infective dose, and the parasite dose required to cause infection in salmonids from the Klamath River, USA, where *C. shasta* is endemic. Klamath River redband rainbow trout (and anadromous steelhead, *Oncorhynchus mykiss*) are highly resistant to infection (Bartholomew et al. 2001) and were not included in this study. In contrast, Klamath River Chinook salmon *Oncorhynchus tshawytscha* and coho salmon *O. kisutch* succumb to infection when parasite abundance is high (Foott et al., 1999; 2004; Stocking et al., 2006). Thus, determining the infective dose is critical to interpreting and predicting disease effects on these valued stocks.

MATERIALS AND METHODS

Polychaete infection and actinospore collection: A colony of *M. speciosa* is maintained at the John L. Fryer Salmon Disease Laboratory (SDL), Oregon State University, Corvallis, OR, USA. To establish the colony, polychaetes and associated sediment were collected with a plankton net from the Klamath River Basin, OR and CA in April 2005, April and August 2006, and May 2007. Samples were placed in coolers supplied with oxygen using an airstone and transported at ambient river temperature. Polychaetes were maintained under static conditions with aeration for up to one week at the laboratory prior to transfer to an aquarium (2005 only) or circular flow-through tanks. The aquarium was supplied with specific pathogen free (SPF) well water and oxygen. Flow-through tanks received untreated Willamette River

water as a source of nutrients. Water temperature in these tanks was not controlled and changed seasonally.

To infect *M. speciosa,* rainbow trout intestinal tissues containing *C. shasta* myxospores were added to the tanks. To maintain a high *M. speciosa* infection prevalence, infected material was added as it became available; weekly to bi-weekly for several months. Additionally, the Willamette River supports the *C. shasta* life cycle; therefore, the untreated river water may have added myxospores to infect the colony as well. Twelve weeks after the first addition of myxospores, 10 ml sub-samples of sediment containing the polychaetes were removed and examined regularly until infected worms were detected. Dental hooks were used to separate polychaetes from the sediment and their tubes under a dissecting microscope at x60 magnification. Individual polychaetes were examined under a light microscope at x200 in a drop of water under a cover slip and infection was confirmed by the visualization of actinospores.

Actinospore quantification: Actinospores were released from polychaete tissue by applying pressure to the worm on a slide with a cover slip. Mature, intact actinospores (characterized as having a tetrahedral shape and 3 unfired polar capsules) were enumerated by averaging the counts of three 5 µl aliquots. Actinospores were either used the same day or stored at 4°C and used within 24 h of collection.

Fish challenges: Rainbow trout fry were obtained from Trout Lodge (Winema, WA) and reared at the SDL on SPF well water. Trout Lodge fish are not from an endemic river basin and are highly susceptible to ceratomyxosis. Klamath River strain Chinook and coho salmon were obtained from Iron Gate Hatchery (Hornbrook, CA), transported to the SDL and reared on SPF well water until the time of challenge. *Ceratomyxa shasta* is endemic to the Klamath Basin, and these stocks have evolved a degree of resistance. For all experiments, the actinospores were added to a plastic container of SPF well water at the appropriate experimental temperature: 13°C for rainbow trout and coho salmon, 13 and 18°C for Chinook salmon. Water baths were used to maintain temperatures in Chinook salmon challenges. Water in the container was mixed to distribute actinospores and oxygen was supplied with an

airstone. All challenges were for 2 h, after which fish were transferred to 25 l flow-through tanks supplied with SPF well water at the appropriate experimental temperature. A sham exposure served as a negative control for each of the challenges.

To determine the infective dose and how factors such as actinospore concentration and fish size affect infection prevalence and severity, rainbow trout were challenged in three infection experiments. The infective dose for Chinook and coho salmon was tested in two additional separate trials. The availability of actinospores was unpredictable; therefore, experiments could not be conducted simultaneously and are described below.

Experiment 1: To determine the infective dose for susceptible rainbow trout, 3 replicate groups of 10 fish (mean ± SD: 0.6 ± 0.3 g) were challenged with 1, 5, 10, 20, 40, or 100 actinospores per fish in 0.5 l at 13°C.

Experiment 2: The relationship between parasite concentration (equal parasite doses in different exposure volumes) and infective dose was tested by challenging 3 groups of 10 rainbow trout fry (mean 0.22 ± 0.05 g) with 1, 5, or 10 actinospores per fish in 1 l at 13°C. These doses were chosen to provide a comparison to the 0.5 l challenges in Experiment 1. In a second trial, 10 fish (mean 6 ± 2.5 g) were challenged individually with 20 actinospores at 2 concentrations; 5 fish were challenged in a volume of 0.5 l (equivalent to 40 spores l^{-1}) and 5 fish in 3 l (equivalent to 6.7 spores l^{-1}).

Experiment 3: To determine the effect of fish size on infective dose, 5 rainbow trout *Oncorhynchus mykiss* averaging 85 ± 20.5 g ("large") were individually exposed to 20 actinospores in 3 l at 13°C. This was run in parallel with the second challenge of Experiment 2 ("small" fish) providing simultaneous comparison of "large" and "small" fish to 6.7 spores l^{-1}.

Experiment 4: Chinook salmon were challenged to a range of doses and at two temperatures for the two highest doses. Groups of 5 juvenile salmon (average 1.7 ± 0.22 g) were each challenged to 10, 50, 100, or 500 spores per fish in 1 l at 13°C. Challenges were conducted in triplicate except for the 500 spore group which was done in duplicate. To test the effect of temperature on the dose response, individual salmon (average 2.3 ± 0.25 g) were challenged to 1,000 or 5,000 actinospores in 1 l at

13 and 18°C. In total, 10 fish were individually challenged to each of the experimental doses at each of the experimental temperatures. Fish were then reared at the experimental temperature for the duration of the experiment.

Experiment 5: Based on results of the Chinook salmon challenge, coho salmon (mean 5.6 ± 1.8 g) were challenged only at the higher parasite doses in 1 l at 13° C. Ten fish were individually challenged to 500 and 1,000 actinospores, and 5 fish were challenged with 5,000 actinospores. To verify the infectivity of the actinospores used in Experiments 4 and 5, 5 susceptible rainbow trout were exposed to 20 actinospores per fish in 1 l in parallel to Chinook and coho salmon challenges.

For all challenges: Fish were fed a commercial diet (Bio-Oregon) and monitored for signs of infection and mortality for 90 days after exposure for rainbow trout and 60 days for Chinook and coho salmon. Moribund fish, defined as fish showing clinical signs and unable to maintain equilibrium, were euthanized with an overdose of MS-222 (tricaine methanesulfonate, Argent Laboratories, Redmond, WA, USA) and were recorded as mortalities. A wet mount of a scraping of the intestine was examined for the presence of myxospores for 2 minutes (Bartholomew, 2002). When myxospores were found, the fish was considered infected. If no myxospores were detected, a sample of posterior intestinal tissue was collected and frozen for assay by polymerase chain reaction (PCR) (Palenzuela et al., 1999). All fish surviving the experimental period were euthanized, and intestinal tissue was collected and assayed by PCR.

Statistical analysis: S Plus version 7.0 statistical software was used (Insightful Technologies). One way analysis of variance (ANOVA) with Bonferroni procedure (Experiments 1 and 2), t-tests (Experiment 2), and linear regression (comparison between Experiments 1 and 2) were used to determine significant differences between infection prevalence and mean days to death (MDD) between the treatments.

RESULTS

Infectious dose for susceptible rainbow trout: The mean infection prevalence (± SD) in groups challenged in 0.5 l with 1 spore was 57 ± 5.8%; at all other doses, 100 ± 0% of the fish were infected (Figure 5.1). In the sham exposure, all fish survived to the end of the experiment and all fish were negative by PCR. One fish

from one of the replicates of the 10-spore dose died within the first week after challenge and was not included in the analysis.

The MDD of rainbow trout challenged in 0.5 l decreased as the dose increased (Figure 5.1). There was a significant difference between the MDD of fish challenged with 1 spore and all other doses (one way ANOVA with Bonferroni procedure on all Experiment 1 challenges $p<0.01$). There was no significant difference in the MDD between the 5- and 10-spore doses, although the MDD of fish challenged with 5 spores was significantly different from fish challenged with the other doses. Significant differences also exist between MDD of fish challenged with 10 and 100 spores. Fish that survived to the end of the 90 day experiment (all from the 1-spore dose) were all negative by PCR.

Fish challenged in 1 l with 1, 5, or 10 actinospores per fish also showed increasing infection prevalence as the actinospore dose increased (Figure 5.2). Fatal infections occurred in all of the treatment groups, except for fish challenged with 1 actinospore, where a single fish was infected (positive by PCR) but survived to the end of the experiment. Although the mean prevalence of infection was higher as dose increased, the difference was not significant among fish challenged in 1 l (one-way ANOVA $p = 0.06$). There was no significant difference between the MDD of fish challenged in 1 l (t-test of 5- and 10-spore doses $p = 0.22$).

Relationship between parasite concentration and dose: The mean infection prevalence among fish challenged in 0.5 l was significantly higher than the 1 l challenge at equivalent doses (linear regression with interaction between volume and dose, $p < 0.01$). When fish were challenged in 0.5 l, the mean infection prevalence in groups challenged with 1, 5, and 10 spores/fish was 57 ± 5.8, 100 ± 0 and 100 ± 0 %, respectively. When the same parasite dose was administered in a challenge volume of 1 l, infection prevalence was 3 ± 5.8, 20 ± 17.3 and 49 ± 24.4%, respectively. At each of the doses, the fish challenged in 0.5 l had a lower MDD than fish challenged in 1 l at the same dose (Figure 5.2).

In the second challenge of Experiment 2, the infection prevalence of fish challenged with 20 actinospores in 0.5 l (concentration: 40 spores l^{-1}) was 100 % with 80% mortality, whereas the infection prevalence of fish challenged at the same dose

in 3 l (concentration: 6.7 spores l^{-1}) was 60% with 40 % mortality (Figure 5.3). In 0.5 l, 4 of the 5 fish died, one infected fish survived, and the MDD was 63 ± 13. Only 2 fish had fatal infections in the 3 l challenge, one infected fish survived, and the MDD was 59 ± 6. As there is only one data set for each of the 20 actinospore challenge groups from Experiment 2, no statistical analysis was done.

Relationship between fish size and parasite dose: The prevalence of *C. shasta* in "large" fish challenged to 20 actinospores in 3 l (Experiment 3) was 100% with 80 % mortality, and the MDD was 63. The prevalence of *C. shasta* in "small" fish challenged to 20 actinospores in 3 l (Experiment 2) was 60% with 40% mortality and the MDD was 59 (Figure 5.4). One additional fish from the survivors of each challenge was positive for *C. shasta* DNA by PCR. No statistical analysis was done on this data set because fish were challenged individually.

Infectious dose for Chinook and coho salmon: No Chinook salmon became infected at any of the doses or temperatures tested. There were no fatalities, none showed signs of clinical infection, and none assayed positive by PCR. Rainbow trout that were exposed in parallel became infected in each of the exposure trials.

A fatal infection occurred in one coho salmon challenged with 1,000 actinospores per fish. This fish produced myxospores that were visualized in a wet mount. However, no coho salmon had fatal infections at the higher 5,000 actinospore dose, and none assayed positive by PCR at this dose.

DISCUSSION

Control over *C. shasta* actinospore dose, concentration, and fish size in the laboratory facilitated the determination of the infective dose for susceptible rainbow trout. These fish developed fatal infections when challenged with as few as one *C. shasta* actinospore per fish. Thus, one actinospore is both an infective and lethal dose for this rainbow trout strain. This study confirms the findings of Ratliff (1983), who was unable to quantify actinospores in natural challenges, but nonetheless estimated that one *C. shasta* was capable of causing fatal infection in a susceptible strain of rainbow trout.

However, susceptibility to infection varies within a species, and rainbow trout from endemic rivers such as the Pit (CA), Skamania (WA), Deschutes (OR), and

Klamath River (OR-CA), are more resistant (Buchanan et al. 1983, Bartholomew et al. 2001, (Ibarra et al., 1991; Ibarra et al., 1992b, 1994) and are likely to require a high infective dose. Further evidence for resistance of Klamath River rainbow trout is from 3 day exposures in the Klamath River where native Chinook (Hallett and Bartholomew 2006, Stocking et al. 2006) and coho salmon suffered mortality, yet simultaneously exposed native redband trout and steelhead remained uninfected (author's unpublished data).

Due to the low infective and lethal dose for susceptible rainbow trout, the effects of increasing dose could only be observed at very low doses. Infection prevalence was approximately 50% when exposed to 1 spore and increased to 100% when challenged with ≥5 actinospores (in 0.5 l challenge). The maximum infection prevalence and mortality were achieved at the second to lowest dose (5 spores), which prevents these parameters from accurately describing a dose response. Therefore, the MDD is used as a measure of infection severity, based on the assumption that fish with more severe infections die earlier. In this study, the MDD decreased when dose was increased from 1 to 10 to 100 actinospores. However, there were no significant differences between the MDD at doses greater than 20 actinospores. Therefore, once a fatal dose is acquired, additional parasites do not increase infection severity in terms of MDD. In this study, parasite severity is described only by MDD; histopathology (Bartholomew et al., 2004) and estimating myxospore abundance in affected fish could further clarify the effect of dose on the progress of infection.

Parasite concentration significantly affected infection prevalence. Fatal infections occurred in 100% of the fish challenged with 5 actinospores in 0.5 l water, but in only 20% of the fish challenged in twice this volume. This effect was also evident at a higher infectious dose, although the differences were not as marked. One explanation for this difference is that fish have fewer encounters with the parasite in larger water volumes than in smaller volumes. Fish were exposed to the parasite for only 2 h in this study, and an increase in exposure time may also have resulted in an increase in parasite encounter and subsequently higher infection prevalence. However, a longer exposure would be more indicative of a static system than a

flowing river. Interestingly, when larger fish were exposed to 20 actinospores in 3 l, infection prevalence and mortality mirrored that of smaller fish in the 0.5 l challenge. In this case, the larger fish occupied a higher proportion of the water and were therefore more likely to encounter the parasites.

In natural systems, predicting the effects of dilution on parasite prevalence are much more complex. Factors such as continuous release of actinospores from polychaetes in the system, fluctuating water flow rates and the ability of the parasite to attach to the host under those conditions must be considered. Several studies have noted a decrease in myxozoan parasite infection prevalence or severity in faster water flows (Bjork and Bartholomew 2009, Hallett and Bartholomew 2008, Vincent 2002). Conversely, Foott et al. (2007) reported no decline in fish infection prevalence or parasite concentration in water samples after a three-fold increase in river flow. In the river, increased flow may stimulate actinospores to be released from their polychaete host or increase the rate by which actinospores are transported from an infectious area upstream. Both of these effects may add more actinospores to the river per unit time. In this study, we demonstrated that dilution of the parasite results in decreased infection prevalence; however, parasite dilution in a natural setting is highly dependent on the presence and proximity of an infected polychaete population.

For susceptible rainbow trout, fish size did not affect infective dose. When fish were challenged in a volume proportional to their size (small fish in 0.5 l and large fish in 3 l), infection prevalence and mortality were equal. However, when large and small fish were challenged at the same spore dose in the same volume, the effect of parasite concentration was evident. This age-independent mortality contrasts with the relationship between age and infective dose for another myxozoan parasite, *Myxobolus cerebralis*, in which younger and hence smaller fish are more susceptible to disease (Markiw, 1991; Sollid et al., 2003; Ryce et al., 2004; Ryce et al., 2005). *Myxobolus cerebralis* proliferates in the cartilage of its fish host, and as fry mature and cartilage ossifies into bone, the amount of target tissue decreases. The intestine is the target tissue for *C. shasta*; therefore, it is not surprising that fish size did not affect infection prevalence.

It is interesting that there were no obvious effects of fish size on MDD. Large fish have more intestinal tissue than small fish, which could influence infection severity and the rate of disease. The infection dynamics of *C. shasta,* specifically the location and reproductive rate would determine whether a greater amount of target tissue would affect infection severity. If parasite proliferation is finite, for example one actinospore produces 1000 myxospores; a larger intestine would result in a smaller percentage of the tissue infected compared to smaller fish. If parasite proliferation is unlimited, a larger intestine would provide more host tissue to infect, and parasite proliferation in a larger intestine could exceed that of a smaller one. The latter explanation appears to fit the data from this study. It is also possible that the high susceptibility of the rainbow trout strain used in this study may have masked the relationship between size and infection severity that may be more evident in a less susceptible fish strain.

Studies on the relationship between fish size and susceptibility to infection by other myxozoan parasites are more difficult to interpret because they were not conducted under controlled conditions. An increase in infection prevalence and parasite abundance with increasing fish size was reported for *Enteromyxum scophthalmi* (Quiroga et al., 2006) but not *Kudoa* spp. (Shaw et al., 1997; Wang et al., 2005). Fish in those studies were collected from natural populations or from aquaculture centers, where older, larger fish are likely to have had longer exposure to the parasite in comparison to younger, smaller fish. This increased exposure time presents a size-independent explanation for differences in prevalence and infection intensity. Additionally, these studies are confounded by the exclusion of smaller fish that were infected and died prior to data collection. In our study, there were no differences in infection prevalence between large and small fish; therefore, in susceptible rainbow trout at least, these factors do not appear to play a significant role.

We were unable to determine the infective dose of *C. shasta* for Klamath River Chinook and coho salmon. In contrast to a lethal dose of a single parasite for susceptible rainbow trout, the inability to infect Klamath River salmon with 5,000 parasites highlights the selective pressure this parasite has played in rivers where it is

endemic. The infection of a single coho salmon at a dose of 1,000 actinospores suggests that this fish may have been compromised or represented individual variation in parasite resistance.

Although we were unable to surpass the threshold for infection of Klamath River salmon in the laboratory, our challenges do corroborate estimates of parasite abundance in the Klamath River. Using molecular methods to measure parasite abundance, Hallett and Bartholomew (2006) detected greater than 20 spores per liter of water at some sites. A concurrent 3d exposure of Klamath Chinook salmon resulted in 68.6% infection prevalence and 48.6% mortality (Stocking et al., 2006). This equates to fish being exposed to greater than 1 million spores in a day when water flow (USGS National Water Information System: Web Interface. http://waterdata.usgs.gov Klamath River, Orleans site no. 11523000) is used to extrapolate the dose. Foott et al. (2007b), measured water flow and parasite concentrations, and estimated that in 6 h, nearly 360,000 spores infected 46% of the Klamath Chinook salmon challenged.

The ability of a single parasite to cause mortality in a susceptible rainbow trout suggests that *C. shasta* is a highly efficient pathogen. Bartholomew et al. (1997) describe the actinospore as having one sporoplasm, indicating the presence of one infective germ cell. In contrast, *M. cerebralis* has an infective dose (resulting in clinical signs) of 10 actinospores and a lethal dose of 100 parasites in susceptible rainbow trout (Ryce et al., 2004). *Myxobolus cerebralis* has 64 germ cells in each triactinomyxon (El-Matbouli and Hoffmann, 1998), thus delivering 64 times the infectious material of a *C. shasta* actinospore. *Tetracapsuloides bryosalmonae*, like *C. shasta*, has a small number of infectious sporoplasms and just one parasite can cause fatal infections in non-native rainbow trout (McGurk et al., 2006). Thus, the high pathogenicity of *T. bryosalmonae* and *C. shasta* may compensate for the low number of infective germ cells. This high pathogenicity explains the role of *C. shasta* as a selection factor, driving the evolution of resistance in native stocks.

Although this study is the first to challenge fish to a range of *C. shasta* actinospore doses, there were some limitations. Due to the asynchronous development of actinospores in *M. speciosa*, the number of mature actinospores available to

perform infection experiments could not be determined until worms were sorted and observed. Actinospore collection methods could also be improved, as mechanical collection of actinospores from infected hosts is both time consuming and labor intensive. However, the small size of the *C. shasta* actinospore and the inability to distinguish it from other particles complicates actinospore collection by the filtration methods that have been used successfully for *M. cerebralis*. Both of these factors prevented the simultaneous challenge of all fish at all doses and is the reason for the staggered experiments. Another limitation was the inability to obtain sufficient actinospores to infect Chinook and coho salmon. The number of actinospores collected in this study is small in comparison to the thousands (4.6×10^4) of *M. cerebralis* actinospores collected from a single *Tubifex tubifex* (Gilbert and Granath, 2001). Because of the small size of *M. speciosa* (approximately 1/10 that of *T. tubifex*) and the location of parasite development in the dermis of the polychaete (Bartholomew et al., 1997), there is inherently less area for actinospores to develop and thus fewer spores are produced per worm.

Despite these limitations, we developed a *C. shasta* infection model for fish in the laboratory that will be critical for examining the confounding factors that exist during natural exposures. For *M. cerebralis,* one of the most studied myxozoans, the laboratory infection model facilitated investigation of a multitude of host parasite interactions including the route of infection (El-Matbouli et al., 1995), fish response to various doses (Markiw, 1992; Ryce et al., 2004) and host specific responses to the parasite (Baerwald et al., 2008). In the *C. shasta* host-parasite relationship, these factors have not been thoroughly investigated, but with a laboratory infection model in place (at least for rainbow) they may be addressed in the future.

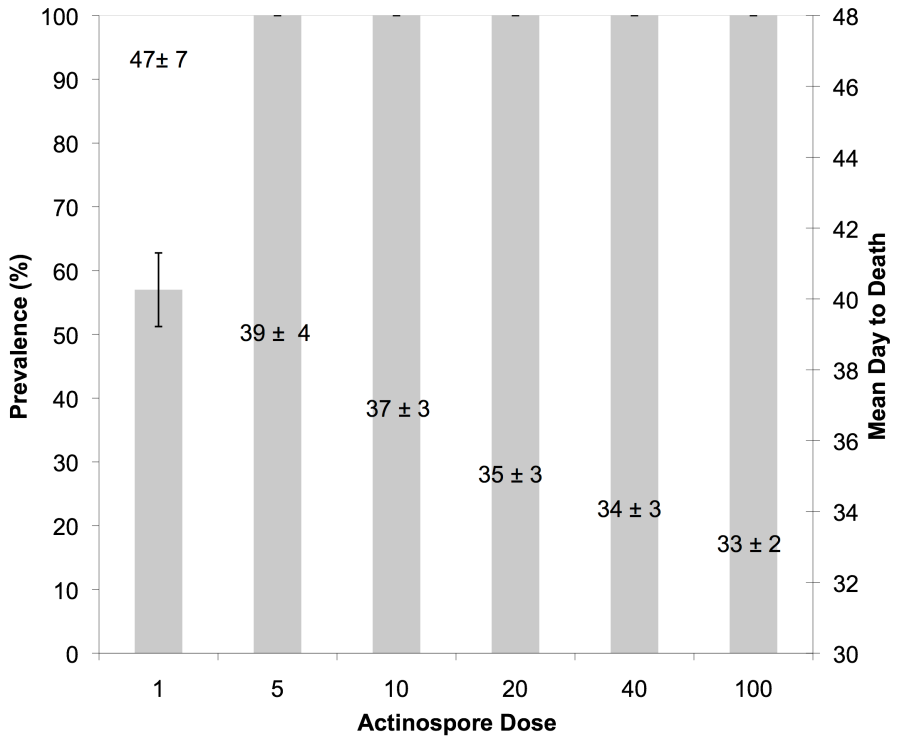

Figure 5.1. Effect of *Ceratomyxa shasta* actinospore dose on infection. The infection prevalence (mean of 3 replicates), mortality and mean day to death of susceptible rainbow trout *Oncorhynchus mykiss* after challenge to a range of actinospore doses (1-100) in 0.5 l.

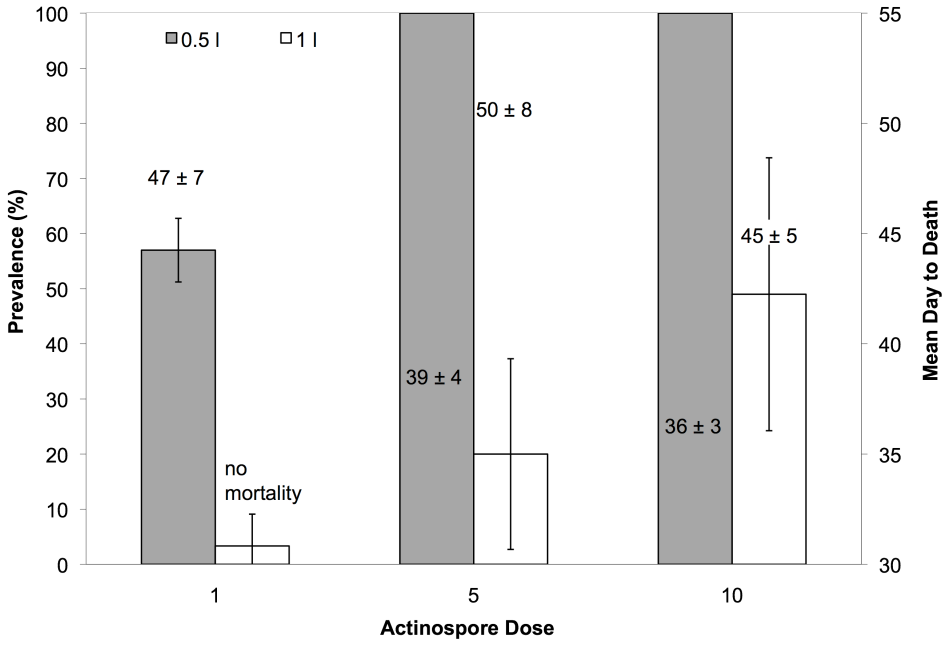

Figure 5.2. Effect of concentration on *Ceratomyxa shasta* infective dose. Infection prevalence (mean of 3 replicates) and mean day to death of rainbow trout *Oncorhynchus mykiss* challenged in 1, 5, or 10 actinospores in 0.5 or 1 l water.

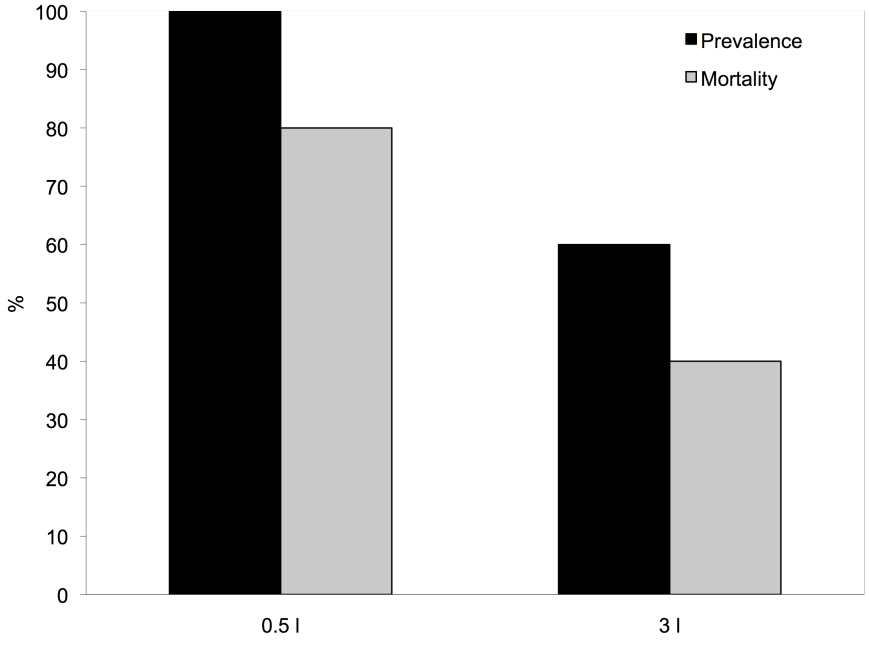

Figure 5.3. Effect of concentration on *Ceratomyxa shasta* infection prevalence. Infection prevalence and mortality of susceptible rainbow trout *Oncorhynchus mykiss* challenged with 20 actinospores in either 0.5 (40 actinospores l^{-1}) or 3 l (6.7 actinospores l^{-1}) for 2 h.

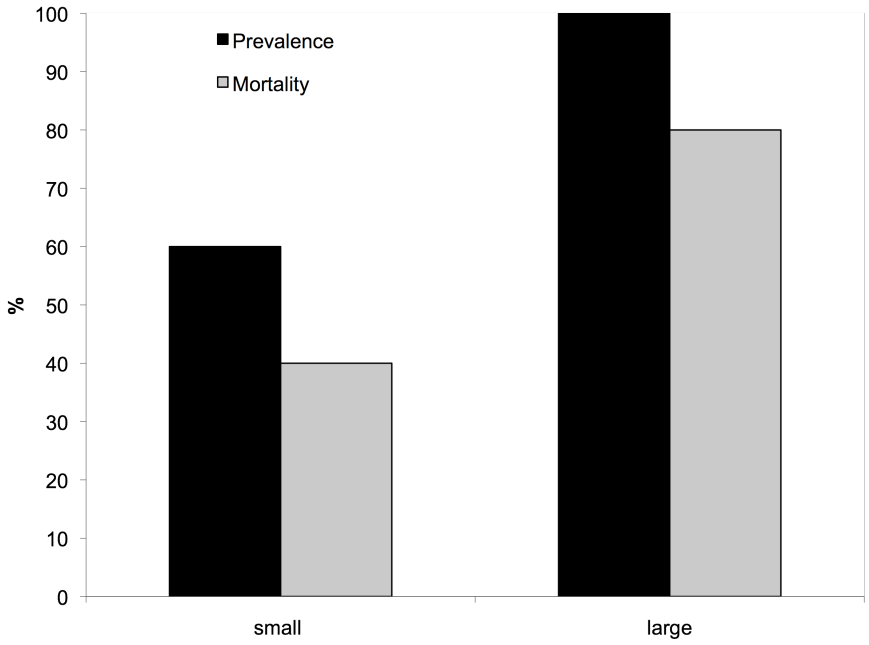

Figure 5.4. Effect of fish size on *Ceratomyxa shasta* infection prevalence. Infection prevalence and mortality of susceptible rainbow trout *Oncorhynchus mykiss* challenged at two sizes, small (6 g ± 2.5) and large (85 g ± 20.5), with 20 actinospores in 3 l (equivalent to 6.7 actinospores l^{-1}).

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ACKNOWLEDGEMENTS

We thank S. Atkinson for initial infected worm specimens; L. Osborne and K. Thames for their help in sorting polychaetes for actinospore challenges; H. Lorz for assistance in setting up fish challenges; D. Stevens for maintaining the flow of river water with plumbing and pump maintenance; R. Stocking and R. Holt assisted in polychaete collection and fish transport; D. Simon is also thanked for assistance in polychaete collection on the Williamson River; and G. Buckles for assistance with PCR. K. Rushton and the staff of Iron Gate Hatchery, CA provided Chinook and coho salmon. We express thanks to the Nature Conservancy for land access to our sampling site. S. Hallett is thanked for comments on manuscript preparation. Funding for this work was provided by the California Energy Commission and Oregon Sea Grant.

CHAPTER 6: INVASION OF *CERATOMYXA SHASTA* (MYXOZOA) AND COMPARISON OF MIGRATION TO THE INTESTINE BETWEEN SUSCEPTIBLE AND RESISTANT FISH HOSTS

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Accepted for publication in International Journal for Parasitology

ABSTRACT

The myxozoan parasite *Ceratomyxa shasta* infects salmonids causing ceratomyxosis, a disease elicited by proliferation of the parasite in the intestine. This parasite is endemic to the Pacific Northwest of North America and salmon and trout strains from endemic river basins show increased resistance to the parasite. It has been suggested that these resistant fish (i) exclude the parasite at the site of invasion and/or (ii) prevent establishment in the intestine. Using parasites pre-labeled with a fluorescent stain, carboxyfluorescein succinimidyl diacetate (CFSE), the gills were identified as the site of attachment of *C. shasta* in a susceptible fish strain. In situ hybridization (ISH) of histological sections was then used to describe the invasion of the parasites in the gill filaments. To investigate differences in the progress of infection between resistant and susceptible fish, a *C. shasta*-susceptible strain of rainbow trout (*Oncorhynchus mykiss*) and a *C. shasta*-resistant strain of Chinook salmon (*Oncorhynchus tshawytscha*) were sampled at consecutive time points following exposure at an endemic site. Using ISH in both species, the parasite was observed to migrate from the gill epithelium into the gill blood vessels where replication and release of parasite stages occurred. Quantitative PCR verified entry of the parasite into the blood. Parasite levels in blood increased 4 days p.i. and remained at a consistent level until the second week when parasite abundance increased further and coincided with host mortality. The timing of parasite replication and migration to the intestine were similar for both fish species. The field exposure dose was unexpectedly high and apparently overwhelmed the Chinook salmon's defenses, as no evidence of resistance to parasite penetration into the gills or prevention of parasite establishment in the intestine was observed.

Keywords: *Ceratomyxa shasta*, Myxozoa, Actinospore, In situ hybridization, Infection, *Oncorhynchus mykiss*, *Oncorhynchus tshawytscha,* CFSE, Parasite resistance

INTRODUCTION

Ceratomyxosis, a disease of salmonids, is characterized by hemorrhage and necrosis of the intestine and is caused by the myxozoan parasite *Ceratomyxa shasta.* This parasite is endemic to the Pacific Northwest, United States of America (USA) (Sanders et al., 1970; Margolis and Evelyn, 1975; Ratliff, 1981; Ching, 1984; Hoffmaster et al., 1988) and has recently been associated with loss of juvenile Chinook salmon (*Oncorhynchus tshawytscha*) in the Klamath River (Foott et al., 2004; Foott et al., 2007a). Klamath Chinook salmon are considered resistant to the parasite (Foott et al., 1999; Stocking et al., 2006) compared with the same species from non-endemic rivers. Thus, this high mortality suggests that factors such as high parasite dose and prolonged exposure to the parasite overwhelm host defenses. A series of studies testing inheritance of the trait determined that resistance is genetically controlled (Ibarra et al., 1992, 1994; Bartholomew et al., 2001; Nichols et al., 2003) yet the underlying mechanism(s) are unknown. Exclusion of the parasite at the site of entry (Bartholomew et al., 1989) and prevention of parasite establishment (Ibarra et al., 1994) have both been proposed as protective responses. To test either hypothesis, the process of host invasion must be understood.

Ceratomyxa shasta has a tropism for the intestine, but the parasite is not detected in this tissue until about 7 days p.i. (Yamamoto and Sanders, 1979; Bartholomew et al., 1989; Fox et al., 2000). The inability to detect *C. shasta* prior to 7 days was considered to be due to low numbers of infecting parasites that may have been missed at earlier sampling times (Fox et al., 2000) or differences in antigenic structure of early parasite stages that prevented their recognition by specific antibodies (Bartholomew et al., 1989). The site of parasite penetration and the route by which the parasite reaches the intestine are unknown due to the lack of diagnostic tools that can detect all parasite life stages and the complex life cycle of *C. shasta* (Bartholomew et al., 1997). The life cycle of *C. shasta* involves two distinct morphological life stages: the actinospore and the myxospore, and two hosts, a salmonid and the freshwater polychaete *Manayunkia speciosa* (Bartholomew et al., 1997). The actinospore is the stage infectious for the fish, and development in the fish host culminates in the myxospore stage. The recent establishment of a laboratory

infection model of *C. shasta* (Bjork and Bartholomew, 2009) and availability of molecular detection methods that can detect all parasite stages (Palenzuela et al., 1999; Palenzuela and Bartholomew, 2002) permits a re-examination of the route of infection.

This study has three goals: (i) to identify the site of parasite invasion and follow the route of parasite migration to the intestine; (ii) to describe parasite development and morphology during migration; and (iii) to evaluate potential differences in invasion and migration between susceptible and resistant hosts.

MATERIALS AND METHODS

Fish

Susceptible rainbow trout (*Oncorhynchus mykiss*) (Troutlodge, Inc., Sumner, WA, USA), for which the infective dose is very low (Bjork and Bartholomew, 2009), were used in laboratory experiments, where adequate numbers of actinospores for challenges could be obtained, and in field exposures. Comparatively resistant Klamath Chinook salmon (Iron Gate Hatchery, Hornbrook, CA, USA) were used only in field exposures, where a much higher infective dose was possible. *Ceratomyxa shasta* infection is not reported from the Troutlodge or Iron Gate Hatchery and both rainbow trout and Chinook salmon from these sources have consistently been negative for the parasite by histology and PCR (Foott et al., 2004, 2007; Stocking et al., 2006; Bjork and Bartholomew, 2009).

Actinospore collection

Actinospores for the attachment and invasion experiments were harvested from a colony of infected *M. speciosa* (Bjork and Bartholomew, 2009).

Actinospore attachment

To determine the site of parasite attachment, actinospores were labeled with a fluorescent stain prior to infecting fish. To separate actinospores from polychaete tissues, the suspension (total volume less then 500 μL) was filtered as follows: a 2 cm diameter circle of 5 μm Nitex mesh (Cell Micro Sieves, Biodesign, Carmel, NY,

USA) was folded into quarters to form a small funnel and placed in a 2 mL microcentrifuge tube. The actinospore suspension was pipetted into the funnel and the tube was centrifuged at 1,000 rpm (78 x *g*) for 3 min with a soft brake. Actinospores were gently washed off the mesh, re-suspended in 1 mL distilled water and labeled with CFSE [5(6) carboxyfluorescein succinimidyl diacetate, Fluka] following the method described by Yokoyama and Urawa (1997). Briefly, 10 µL of 10 mM CFSE in DMSO was added to the spores and incubated for 15 min at room temperature. The spores were then collected on a 5 µm mesh as described above, re-suspended in distilled water and enumerated. A sub-sample of labeled actinospores was fixed in 1% formalin to assess fluorescence.

Two susceptible rainbow trout fry (0.6 g ± 0.3) were individually challenged with 1,000 labeled actinospores for 30 min in 50 mL specific pathogen-free (SPF) well water at room temperature. Oxygen was provided using an aquarium aerator with a pipette tip attachment. Two unexposed fish were held similarly as negative controls. After 30 min, one control and one challenged fish were euthanized with 200 mg MS222 (tricaine methanosulfonate, Argent Laboratories, Redmond, WA, USA) in 1 L of water, fixed in 2% paraformaldehyde and transferred to PBS. Wet mounts of fins, skin and gills from the fixed fish and the formalin-fixed actinospores were examined using a microscope with a 450-490 nm wavelength filter. To confirm that the labeled actinospores were infective, the remaining challenged positive control fish was reared in 20 °C SPF well water and monitored for *C. shasta* infection. The negative control fish was held under the same conditions and euthanized after 60 days. PCR (Palenzuela et al., 1999) was used to verify that the control fish was negative for *C. shasta*.

Parasite invasion

To examine invasion of *C. shasta* in the gill tissue, two rainbow trout fry (mean 0.6 g ± 0.3) were anesthetized (MS222 100 mg/ L buffered with sodium bicarbonate) and a suspension of 2 x 10^3 actinospores in 100 µL of water was pipetted under the operculum onto the gills of each fish, with each gill set receiving approximately 50 µL. Fish were placed in 120 mL of well water with aeration to

recover. One fish was euthanized after 5 min, fixed in neutral buffered formalin (NBF) and processed by routine methods for histology. In situ hybridization (ISH) (see below) was used to identify the parasite in 5 μm histological sections. To verify infectivity of the actinospores, the remaining fish was transferred to a tank with SPF water at 18° C and monitored for the development of infection. The control fish was euthanized after 60 days and PCR (Palenzuela et al., 1999) was used to verify that the control fish was negative for *C. shasta*.

Infection of susceptible and resistant fish hosts

To attain a dose of *C. shasta* high enough to infect Klamath Chinook salmon, both juvenile rainbow trout (40 fish averaging 2.6 g ± 1.1) and Chinook salmon (40 fish averaging 4.3 g ± 1.4) were held in cages in the Klamath River at a site where parasite densities are high (above Beaver Creek, CA, USA; river km 259.1) (Hallett and Bartholomew, 2006; Stocking et al., 2006). Fish were exposed in June 2008 for 24 h, then fish were transported to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis, OR, USA and reared on 18°C SPF well water in 25 L flow-through tanks. Total exposure dose was estimated to be 5.35×10^8 spores (R. Adam Ray, personal communication). A bath treatment of furanase (Aquarium Products, Glen Burnie, MD, USA) was administered and fish were fed antibiotic-medicated food to minimize bacterial infections. Beginning at 24 h (1 day p.i.) and continuing daily for 7 days, two fish were euthanized with an overdose of MS222. The caudal fin was severed, blood was collected from the caudal vein using non-heparinized capillary tubes (Rowley, 1990) and transferred to a microcentrifuge tube. Due to the small size of the fish, the volume of blood collected was generally ≤ 30 μL, therefore the glass capillary tube containing residual blood was snapped off and included in the tube with the blood. The entire sample was frozen for up to 4 months at -20°C before DNA extraction. After collecting blood, each fish was fixed in Davidson's fixative (to reduce the brittleness that resulted from NBF fixation) and then transferred to 70% ethanol after 24 h. After 7 days, two fish were sampled every other day until 14 days; a final two fish sample was taken at 24 days p.i. After fixation and transfer to ethanol, gills, stomach, kidney, heart, brain, spleen, liver,

pyloric caeca, intestine and samples of skin, muscle and fins were dissected and processed by routine histological methods, embedded in paraffin and serial 5 µm sections were adhered to slides for staining.

ISH

ISH was used to label parasite stages in tissues and proceeded as described by Palenzuela and Bartholomew (2002) with the following modifications. The 3' DIG End labeling kit (Roche Applied Science, Indianapolis, IN, USA) was used to generate digoxigenin-labeled oligonucleotide probes. Proteinase K was increased to 50 µg/mL in Tris $CaCl_2$ for 30 min at 37°C to increase probe access to the tissues. The recipe for 20 mL of pre-hybridization solution was modified as follows: 5.6 mL sterile water, 2 mL 20 x SSC (0.3 M sodium citrate, 3 M NaCl pH 7 .0), 0.4 mL 50 x Denhardt's solution (50 mg BSA, 50 mg Ficoll®, 50 mg PVP [polyvinylpolypyrrolidone] in 5 mL water), 4 mL 50 x dextran sulfate (25 g dextran sulfate sodium salt from *Leuconostoc* spp. molecular weight 6,500-10,000 in 50 mL water), 2 mL 10 x PBS (1.4 M NaCl, 27 mM KCL, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 pH 7.5), 1 mL single-stranded salmon sperm DNA, and 5 mL deionized formamide. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO, USA). The denaturation step was also optimized to 90° C for 5 min followed by a 5 min incubation at 85° C. Washing steps were performed at 35°C (instead of 37°C) on a shaking table set to 400 rpm. Only one wash in 0.25 x SSC was used after the 2 x and 1 x SSC washes. After the blocking solution was drained, slides were allowed to air dry for 5 min at room temperature, then heated in a 37°C incubator for 5 min before incubation with the anti-digoxigenin antibody in media modified for optimum hybridization signal at the following concentrations: 0.2% sheep serum, 0.06% triton X-100 in Genius 1 buffer. Sections were counterstained in 1% methyl green. Alternate sections were stained with May-Grunwald Giemsa or Mayer's H & E.

Microscopy

Tissues were examined using a Leica compound microscope and images captured with a DC 500 camera using IM50 software. Measurements were made

using SPOT software version 3.0.4 (Diagnostic Instruments, Inc., Sterling Heights, MI, USA). The location and morphology of parasites in Chinook salmon and rainbow trout was compared.

Real time quantitative PCR (qPCR) blood analysis

The QIAamp DNA Blood midikit (QIAGEN Inc., Valencia, CA, USA) was used to extract DNA from blood samples with the following modifications and volumes. A 1 mL vol. of PBS was added to the blood. A glass rod sprayed with DNA AWAY (Molecular BioProducts, Inc., Thermo Fisher Scientific, Inc., Waltham, MA, USA), wiped with a paper towel and allowed to air dry, was used to crush the capillary tube. The rod was cleaned as described between each sample. The blood/PBS suspension was transferred to a sterile 10 mL tube, leaving the glass debris. Thereafter, the QIAmp DNA Blood midikit protocol was followed using the protocol for up to 1 mL of whole blood (1.2 mL buffer AL [lysis buffer, QIAGEN catalog number 19075], 100 µL Protease, and 1 mL ethanol). DNA was eluted in 200 µL, reapplied to the column and eluted again to improve DNA concentration. *Ceratomyxa shasta* DNA was quantified using a qPCR assay (Hallett and Bartholomew, 2006). Each sample was assayed in duplicate wells through 40 cycles in the qPCR assay using an ABI 7300 Real-time PCR system on MicroAmp ® optical 96 well plates. If the S.D. of the duplicate wells for an individual sample was >1, the sample was assayed again. Two internal positive controls (DNA from infected fish tissue and an artificial parasite DNA template) and a no template control (NTC, molecular grade water) were included in each assay. The same positive control was used on each qPCR plate, permitting analysis of inter-assay variation. The quantity of blood collected varied between fish, depending on fish size and progress of disease. To avoid sample bias due to these factors, all of the blood collected from the caudal fin was processed. This quantity varied between approximately 10 to 30 µL (one to three capillary tubes). All of the blood collected was included in the extraction for each fish, so the quantitative cycle (Cq) (Bustin et al., 2009) value relates to relative parasite DNA in blood. Only two fish were sampled at each time point for each species, therefore no statistical analysis was performed on differences between

parasite abundance over time. Only samples for which both wells fluoresced were considered positive for parasite DNA. Samples in which parasite DNA was undetected after 40 cycles were assigned a Cq value of 40 for analysis purposes. Assigning these negative samples a value permits the average Cq value of the two fish to be calculated, decreasing the bias that would occur if only positive fish with a Cq value were included.

To evaluate the effect of differing quantities of blood on qPCR detection, six spiked blood standards were tested. One actinospore dried onto a piece of glass coverslip (Hallett and Bartholomew, 2006) was added to each of three tubes containing 10 µL of fish blood and three tubes containing 30 µL of fish blood. Sample DNA was extracted and quantified as described above. A *t*-test was used to evaluate the Cq values obtained from the standards (S- Plus version 7.0 statistical software, Insightful Technologies, Seattle, WA, USA).

The protocol for the use of the animals in this study (Animal Care and Use Protocol 3850) was reviewed and approved by the Institutional Animal Care and Use Committee of the Office of Research Integrity at Oregon State University, Corvallis, OR, USA.

RESULTS

Actinospore attachment

Ceratomyxa shasta actinospores fluoresced brightly after incubation with CFSE (Fig. 1B). Recognizably triangular shaped actinospores and amoeboid sporoplasms with two nuclei were observed attached to both the tip and base of the gill lamellae (Fig. 1A and C). No actinospores were detected on the fins, fin bases or other skin surfaces examined. The fish reared at 20° C after CFSE-labeled actinospore challenge died 15 days after exposure and mature myxospores were detected in a wetmount preparation of intestinal material. The control fish did not develop infection and was negative by PCR.

Parasite invasion

Parasites labeled with the dark purple hybridization signal were detected on the surface of the gill filaments or penetrating the gill epithelium of fish sampled 5 min p.i. (Fig. 2A). At this time the parasite was intercellular, amoeboid and two nuclei were present (Fig. 2B). The positive control fish developed ceratomyxosis and died 20 days p.i. No parasites were observed in the negative control fish and it was negative by PCR.

Parasite migration and comparison between susceptible and resistant fish hosts

All fish challenged in the Klamath River became infected with *C. shasta.* Mortalities in Chinook salmon began on day 16 p.i. and mortalities in rainbow trout began on day 22 p.i. Continued mortality in both species limited the number of fish surviving to be sampled at later dates. After day 24, only two rainbow trout remained, and they both succumbed to infection on day 26, terminating the experiment. The location and morphology of the parasite was similar in rainbow trout and Chinook salmon, therefore the path of migration summarized here applies to both species. Due to the small sample size, statistical analysis was not applied to determine whether timing of parasite migration between the fish species was different; however, minor differences in the timing of the progress of infection are noted. No parasites were detected in the control fish.

At 1 and 2 days p.i., parasite stages were present in the blood vessels of the gill filaments (Fig. 2C and D). From 3 days p.i. and thereafter, parasites were seen in the blood vessels of the gill arches as well (Fig. 3). Parasites in gill blood vessels ranged from 19-41 μm in length, 10-16 μm in width, and were predominantly ellipsoid with one flattened edge in contact with the blood vessel wall. Parasites appeared to be beneath the endothelium (Fig. 3C), however it is not clear whether they were intracellular or at the interface of the endothelium and connective tissue.

Five distinct pre-sporogonic developmental stages were observed in the blood vessels of the gills. Terminology referring to the stages of *C. shasta* in this study are based on descriptions in Lom and Dyková (2006). The first stage, observed starting on day 1 p.i., occurred predominantly in gill filament blood vessels. In this stage, a prominent nucleolus (average diameter 4 μm) is present, formed presumably through

autogamy (Noble, 1941) of the two nuclei of the invading sporoplasm. The nucleolus of this zygote (trophozoite or primary cell) is surrounded by a distinct halo (Fig. 2D) and was observed alone or with smaller secondary cells that contained one larger secondary nucleus and up to two smaller tertiary nuclei (Figs. 2D, 6). In the second stage, also observed beginning day 1 p.i., the primary nucleolus is present in addition to an increased number of secondary cells, each containing a nucleus with average diameter 1 μm (Fig. 3A and B). Smaller vegetative nuclei are present in the cytoplasm of the primary cell (Fig. 3B). The third stage, detected on day 3 p.i., is characterized by darkening and enlargement of the secondary cells (3-4 μm diameter) (Fig. 3C inset). The primary nucleus is enlarged (Fig. 3C inset). In the fourth stage, observed on day 5 p.i., the primary nucleus is absent and up to eight densely staining secondary cells (4-5 μm diameter), each containing a 2 μm lightly stained center surrounding a nucleus, fill the spindle shaped primary cell (Fig. 3D). One to two additional smaller nuclei are observed in these secondary cells (Fig. 3D inset). On day 5 p.i., mature secondary cells, the fifth stage, were detected in the lumen of the gill arch blood vessel (Fig. 3F). All five stages were present in the gill filament and gill arch blood vessels throughout the remainder of the infection.

Also at 4 and 5 days p.i., parasite stages were observed in blood vessels supplying the intestine and in the intestinal serosa (Fig. 4A and B). The cells in the blood vessels had either a spherical morphology similar to the mature secondary cells previously described or were in contact with the endothelium similar to the stages observed in the gills. The parasites had an amoeboid morphology in the adipose tissues superficial to the intestine and between cells of the serosa, muscularis and lamina propria from 4 days p.i. onwards (Fig. 4A-C).

In the intestinal villi, foci of multiple replicative stages were first evident at 5 days p.i. in the lamina propria and epithelium (Fig. 4D). The parasites in the intestinal villi were irregular in shape, variable in size and the enclosed pansporoblasts were in multiple stages of division and differentiation into sporoblasts and sporonts (sporogony) (Yamamoto and Sanders, 1979). The parasites also had a translucent zone surrounding the pansporoblasts. Parasite proliferation progressed from infection of the intestinal epithelium with pansporoblasts 1 week p.i., to diffuse sporogenesis

occurring through all layers of the intestine by 2 weeks p.i. in both fish species (Fig. 3E and F).

Parasite development in other organs, such as the liver and pyloric caeca, occurred after parasite proliferation in the intestine. Parasites were first detected in the pyloric caeca and liver of Chinook salmon on days 5 and 7, respectively, and in both tissues in rainbow trout on day 11. Parasites were not observed in the kidney until 7 days p.i. in Chinook salmon and 11 days in rainbow trout. Parasites were found in the capsule of the spleen on day 24 p.i. but not in the inner cortex, even late in the infection. Parasite stages were not observed in the heart, stomach, brain, skin, fins or buccal cavity.

qPCR blood analysis

The average Cq for 10 µL blood spiked with one actinospore was 36.4 and the average Cq for the 30 µL spiked standards was 37.5. One of the replicates from the 30 µL spiked standards was undetected in both wells and was excluded from analysis. There was no significant difference in the Cq values obtained from the 10 and 30 µL blood samples spiked with one actinospore (*t*-test, $P = 0.096$), therefore the experimental samples are comparable within the range of blood volumes collected. The NTC was undetected in all plates, and the Cq value of the positive controls had a S.D. of 0.40 for tissue and 1.36 for the artificial template between qPCR plates. The highest Cq value of any of the one actinospore standards was 38.2, and was designated the cut-off for positive samples.

In both Chinook salmon and rainbow trout, *C. shasta* DNA was detected in the blood at low levels (Cq values > 35.5) in the first 3 days p.i. (Fig. 5). Parasite DNA in the blood of both fish species increased on day 4 (Cq values range between 32.0 and 33.9) and remained at this level through day 9 (Cq range: 32.4 to 35.9). On days 11-13, parasite DNA in rainbow trout decreased (Cq range: 36.7 to 35.0), then increased (Cq range 32.1 to 25.2), coinciding with fish mortality. A similar trend occurred in Chinook salmon. There was less parasite DNA on day 13 (Cq 36.2) than on day 11 (Cq 33.0), but from days 15 to 24 DNA quantity increased (Cq values 28.4

and 29.2, respectively). Based on the doubling principle of PCR, a 3.3 change in Cq value corresponds to a 10-fold change in DNA quantity.

DISCUSSION

In this study, the gills were identified as the site of attachment of *C. shasta* actinospores. Within 24 h, the parasite penetrated the gill epithelium and migrated to the gill blood vessel, beginning its next developmental phase by producing secondary cells. These cells either migrate into the intestine or continue to multiply in the blood vessels. In the intestine, the secondary cells migrate to the mucosal epithelium, continue proliferating and begin sporogenesis as described by Yamamoto and Sanders (1979).

Previous studies have documented the presence of *C. shasta* in the blood (Noble, 1950; Bartholomew et al., 1989). However, we provide evidence that this blood stage precedes entry to the intestine and that the proliferation of secondary cells occurs in this location early in the infection. Analysis of blood by qPCR revealed an increase in parasite DNA that coincided with these histological observations. During the first 2 weeks, blood of infected fish may have contained 10 to 100 parasites, and as many as 1,000 to 10,000 at 24 days p.i. when extrapolated from the one actinospore standard.

Comparison of early invasion of *C. shasta* into the gills of Chinook salmon revealed no differences compared with rainbow trout, and pathogenic effects of the parasite in the intestine of both species were also similar. High mortality in rainbow trout was expected for all experiments because the lethal dose for this susceptible strain is as low as one actinospore (Bjork and Bartholomew, 2009). However, the high mortality and low mean day to death in Klamath Chinook salmon was surprising because this stock has shown higher resistance to *C. shasta* (Stocking et al., 2006). An explanation for this is that the high parasite dose in the present study (9.2×10^6 parasites per fish, personal communication R. Adam Ray) overwhelmed the resistance mechanisms of the Chinook salmon. Therefore, resistance of parasite entry into the gills and intestine may still play a role at lower parasite doses.

The proliferation of *C. shasta* in organs other than the intestine is well documented (Noble, 1950; Wales and Wolf, 1955; Schafer, 1968; Johnson, K.A. 1975 Host susceptibility, histopathologic and transmission studies on *Ceratomyxa shasta,* a myxosporidian parasite of salmonid fish. Doctoral Thesis, Oregon State University, Corvallis, OR, USA; Bartholomew et al., 1989), but was observed in this study only after *C. shasta* was established in the intestine. Our data confirms the speculation of Bartholomew et al. (1989) that *C. shasta* reached these organs through the blood. However, within the circulatory system, *C. shasta* would have access to these organs early in the infection (4 days p.i.), yet invasion of the liver and kidney did not occur until 5 days in Chinook salmon and 11 days in rainbow trout. Thus, we consider two potential scenarios for *C. shasta* dissemination from the blood. In the first, the intestine is the preferred tissue for *C. shasta*, but the host immune system weakens in severe infections and the parasite is triggered to start sporogenesis regardless of location in the host. Thus, secondary cells in the blood vessels would invade the nearest tissue and begin sporogenesis, but sporogony may also occur in the blood as observed by Noble (1950). In other myxosporeans for which developmental blood stages have been identified (Belem and Pote, 2001; Holzer at al., 2003) parasite stages were detected in other tissues, but sporulation occurred only in the target tissue, supporting the hypothesis for tissue tropism. Alternatively, invasion of other organs occurs by coincidence when extrasporogonic parasite stages occlude the smaller blood vessels and capillaries. These occlusions would lead to rupture of the vessel or localized necrosis, both of which would facilitate dissemination of the parasite into adjacent tissues. Both scenarios maximize parasite proliferation by invading additional host tissues.

Complex migration patterns with portals of entry different from the target tissue and several distinct developmental stages in the fish host are common to the Myxozoa. *Myxobolus cerebralis*, one of the most intensively studied myxozoans, penetrates the epidermis and/or epithelium (skin, fins, gills and buccal cavity) of rainbow trout, burrows through the connective tissue, then travels along the peripheral nerves to the cartilage where it proliferates and matures into a myxospore (Wolf and Markiw, 1984; El-Matbouli and Hoffmann, 1989; El-Matbouli et al., 1992,

1995, 1999; Hedrick et al., 1998). *Myxobolus arcticus* enters through the epidermis of masu (*Oncorhynchus masou*) and sockeye (*Oncorhynchus nerka*) salmon but develops in the brain, whereas *Thelohanellus hovorkai* enters through the gill epithelium of carp (*Cyprinus carpio*) but causes hemorrhage on the fishes' body surface (Yokoyama and Urawa, 1997). *Henneguya ictaluri* actinospores penetrate the gut epithelium and the gills of channel catfish (*Ictalurus punctatus*); however, proliferation and pathological effects occur predominantly in the gills (Belem and Pote, 2001). *Tetracapsuloides bryosalmonae* enters through the gills of rainbow trout (Morris et al., 2000) and skin (Longshaw et al., 2002) but causes disease in the kidney. *Sphaerospora truttae* invades the epithelium of the gills of Atlantic salmon (*Salmo salar*), proliferates in the blood then enters the kidney (Holzer et al., 2003). Although the morphology of *C. shasta* in the blood and the association of developmental stages in the endothelium are most similar to developmental stages of *Sphaerospora* spp. (Lom et al., 1983a, b, 1985; Baska and Molnár, 1988), these species infect different tissues and phylogenetic analyses show that they are not closely related to *C. shasta* (Fiala, 2006). However, the timing of entry into the vascular system (approximately 3 days p.i.) was similar for both *C. shasta* and *S. truttae*, and may indicate a minimum development time for myxozoans that enter the blood from the gills.

Sites for entry of more closely related *Ceratomxya* spp. have not been described, and descriptions of parasite development are primarily of sporogonic stages. However, the secondary cells of *C. shasta* observed in this study closely resemble early sporogonic stages of other *Ceratomyxa* spp. Secondary cells described in this study are morphologically similar to the earliest stage (zygote) described for the sporogonic phase of *Ceratomyxa blennius* (Noble, 1941). The pre-sporogonic development of *C. shasta* in the blood vessel also resembles the schizogonic stages described for *C. blennius* (Noble, 1941). However, we speculate that secondary cells are released by rupture of the primary cell, rather than by external budding. Pre-sporogonic stages of *C. shasta* observed in blood vessels contained numerous secondary cells, similar to *Ceratomyxa sparusaurati* plasmodia (Sitjà-Bobadilla et al., 1995). However, the plasmodium-like structure of *C. shasta* is a pre-sporogonic

developmental stage, whereas *C. sparusaurati* develops true plasmodia (Lom and Dyková, 2006) from which myxospores develop in the gall bladder (Sitjà-Bobadilla et al., 1995). Sitjà-Bobadilla et al. (1995) also observed secondary and tertiary cells that were non-sporous and hypothesized that these cells enhanced proliferation of the parasite and started new sporogonic cycles, which parallels the developmental phases of *C. shasta* that we observed. Morris and Adams (2009) propose that the tertiary cell occurs at the onset of sporogony but in *C. shasta* tertiary structures develop prior to migration to the site of sporogony. These observations indicate that the tertiary cell may have a more variable role in *Ceratomyxa* spp. Ultrastructural analysis of the pre-sporogonic stages of *C. shasta* would facilitate a better characterization of the secondary cell and tertiary structures, and further clarify the development of this parasite.

Of the myxozoans for which complete life cycles have been described, no correlation between morphological features of the actinospore, use of the gill as portal of entry into the fish or similarities based on phylogenetic clustering can be drawn. However, many of the species that enter through the gill also disseminate (or are hypothesized to) to their respective target tissue via the blood (Belem and Pote, 2001; Longshaw et al., 2002; Holzer et al., 2003). In the context of host-parasite co-evolution, this indicates parasite adaptation towards i) minimal migration distance to gain access to the internal environment of the host (only one cell layer in gill epithelium), ii) immediate access to the circulatory system in which dissemination to any tissue is readily available, and iii) conservation of metabolic energy through reliance on the host to disseminate the parasite to the target tissue through normal blood circulation.

Multiple techniques were used in this study and all successfully provided information used to determine the route of infection. CFSE labeling (Yokoyama and Urawa, 1997) facilitated the observation of actinospore attachment without affecting infectivity. ISH (Palenzuela and Bartholomew, 2002) confirmed the findings from the CFSE experiment and facilitated discrimination of the parasite regardless of morphology and location in host tissue. One limitation to this technique was that in tissues with high host cell density (intestine), or high parasite density (large numbers

of trophozoites as occurs late in infection), parasite signal was not as intense as in less dense tissues (gills). For parasite detection alone, PCR and qPCR remain more sensitive than histology. However in this study, visualization of the parasite was critical for describing location and morphology. qPCR permitted comparison of relative parasite concentration in the blood over time and between species, and reinforced histological findings. However, techniques such as electron microscopy are required to resolve the question of inter- or intracellular development of *C. shasta* in the blood vessel as well as provide a more detailed description of parasite development.

In conclusion, the route of parasite invasion and migration was identified and a new developmental stage in the blood was described, permitting previous hypotheses to be refined and new ones to be generated. Ibarra et al. (1991) and Foott et al. (2007b) observed that granulomas in resistant fish contain the parasite, preventing *C. shasta* from establishing and proliferating in the intestine. Our study indicates that this occurs in the latter stages of infection, after proliferation in the blood. We also provide evidence that the presence of *C. shasta* trophozoites in the intestinal lumen of resistant fish (Bartholomew et al., 2004) is a result of migration through the intestine rather than a blocked site of entry as previously suggested. Previous studies also suggest that specific immunity may play a role (Bartholomew et al., 1989; Ibarra et al., 1991) and this remains unresolved. In the present study, neither the gills nor the intestine of Chinook salmon appeared to provide a barrier against parasite penetration or effectively contain the parasite, likely due to the high exposure dose. Further studies at lower parasite doses should focus on the role of the gill in resisting parasite invasion, the role of the blood in eliminating parasite abundance during the migratory phase of *C. shasta*, and the ability of the intestine to contain, eliminate or neutralize the damage induced by the parasites.

ACKNOWLEDGEMENTS

The authors sincerely thank R. Adam Ray and Rich Holt for their assistance with field exposures as well as Fisher's RV Park (Klamath River, CA,USA) for land access to our field site. R. Adam Ray also estimated the parasite dose based on water

samples and velocity measurements and has approved of the use of these estimates in this article. Additionally, we thank Don Stevens, Harriet Lorz and Charlene Hurst for help in maintaining polychaetes in the laboratory as well as with fish care and sampling; Gerri Buckles for qPCR assay, Dolly Baxa of the Hedrick laboratory at the University California, Davis (USA) for advice in optimizing the in situ hybridization protocol, and Sascha Hallett for reviewing the manuscript. This work was supported by Oregon Sea Grant under award number NA10OAR4170059 from the National Oceanic and Atmospheric Administration's National Sea Grant College Program, U.S. Department of Commerce, and by appropriations made by the Oregon State Legislature. The statements, findings, conclusions and recommendations are those of the authors and do not necessarily reflect the views of these funding bodies.

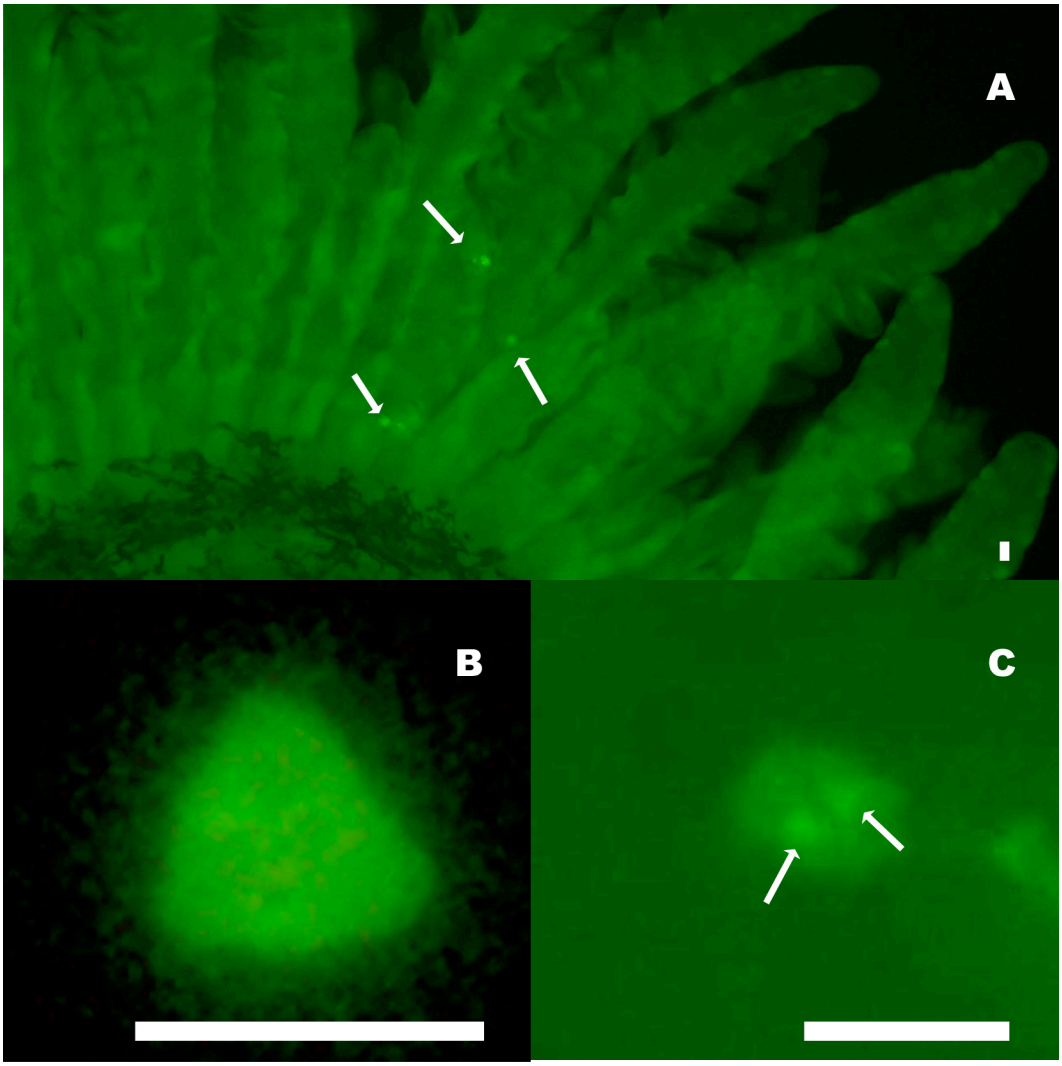

Figure 6.1. CFSE [5(6) carboxyfluorescein, succinimidyl diacetate] stained *Ceratomyxa shasta* actinospores attached to rainbow trout gills 30 min after exposure. Scale bars, 10 µm. A) Wet mount of a gill arch with attached actinospores (arrows). B) Actinospore stained with CFSE. C) Higher magnification of CFSE-stained actinospores attached to gills showing binucleate amoeboid sporoplasm (arrows).

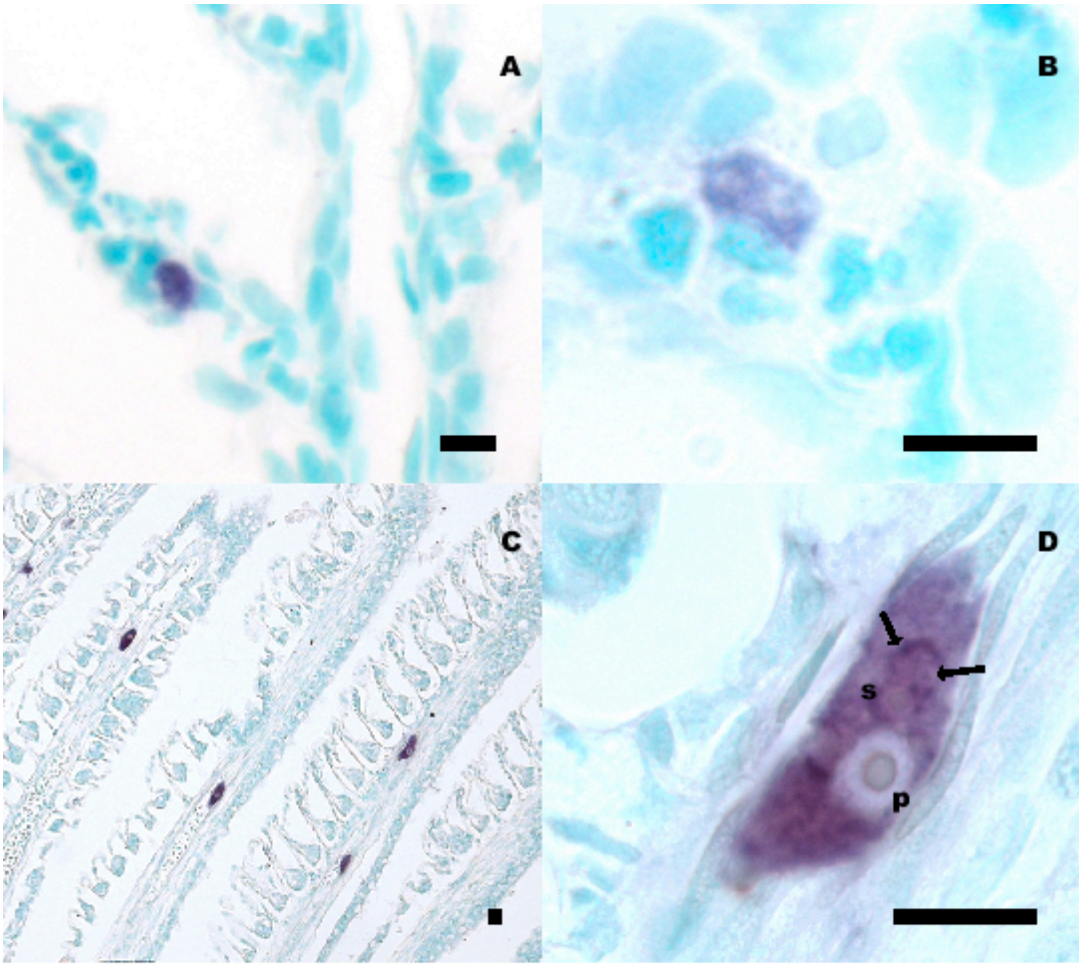

Figure 6.2. In situ hybridized sections of *Ceratomyxa shasta* invading the gills. The parasites are labeled by dark purple stain. Scale bars, 10 µm. A) Sporoplasm on epithelium of gill filament 5 min after exposure to the parasite. B) Intercellular stage of *C. shasta* between epithelial cells of gill filament 5 min after exposure to the parasite. C) Multiple *C. shasta* stages in the blood vessels of the gill filaments 1 day after exposure. D) Amoeboid endothelial-associated stage showing prominent primary nucleolus (p) and a developing secondary cell (s) with tertiary nuclei (arrows) 2 days after exposure.

Figure 6.3. Developmental stages of *Ceratomyxa shasta* in the gills progressing from penetration of the blood vessel to release into the lumen of the blood vessel. Scale bars, 10 µm. A-D and F were stained with Giemsa; E shows in situ hybridization. A) Amoeboid endothelial stage having two secondary cells (s) with tertiary nuclei in addition to primary nucleus (p) 2 days p.i. B) Amoeboid endothelial stage with four secondary cells (s), a primary nucleus (p) and a vegetative nucleus (v) 2 days p.i. C) Amoeboid endothelial stages (arrows) in gill arch. Inset: secondary cell(s) defined and primary nucleus (p) enlarged 5 days p.i. D) Spindle-shaped primary cell containing maturing secondary cells (arrows) in gill arch. Inset: enlarged primary cell containing secondary cells showing multiple nuclei for each cell 5 days p.i. E) Secondary cells rupturing from a primary cell 5 days p.i. F) Mature secondary cells free in the lumen of the blood vessel 5 days p.i.

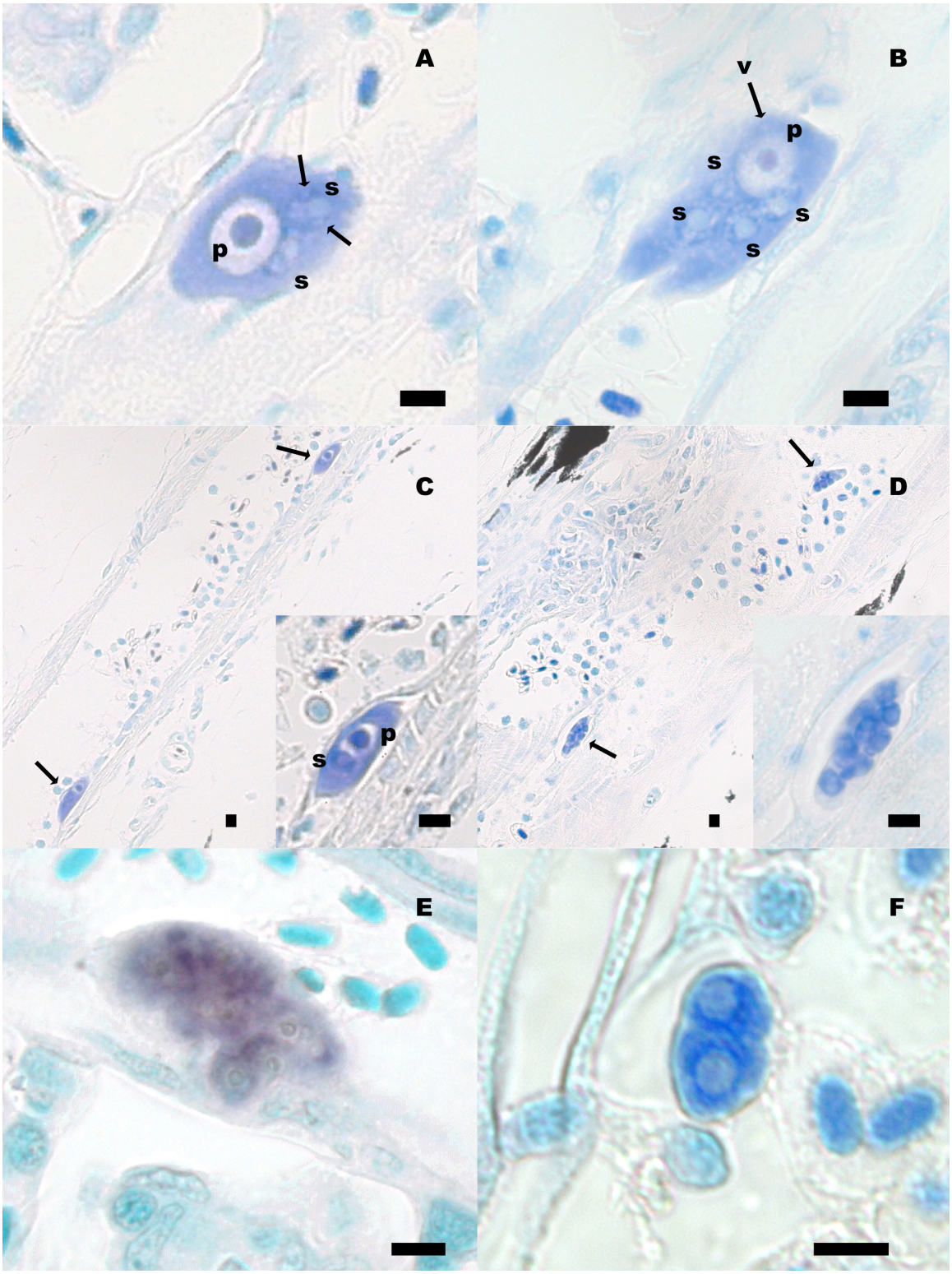

Figure 6.3.

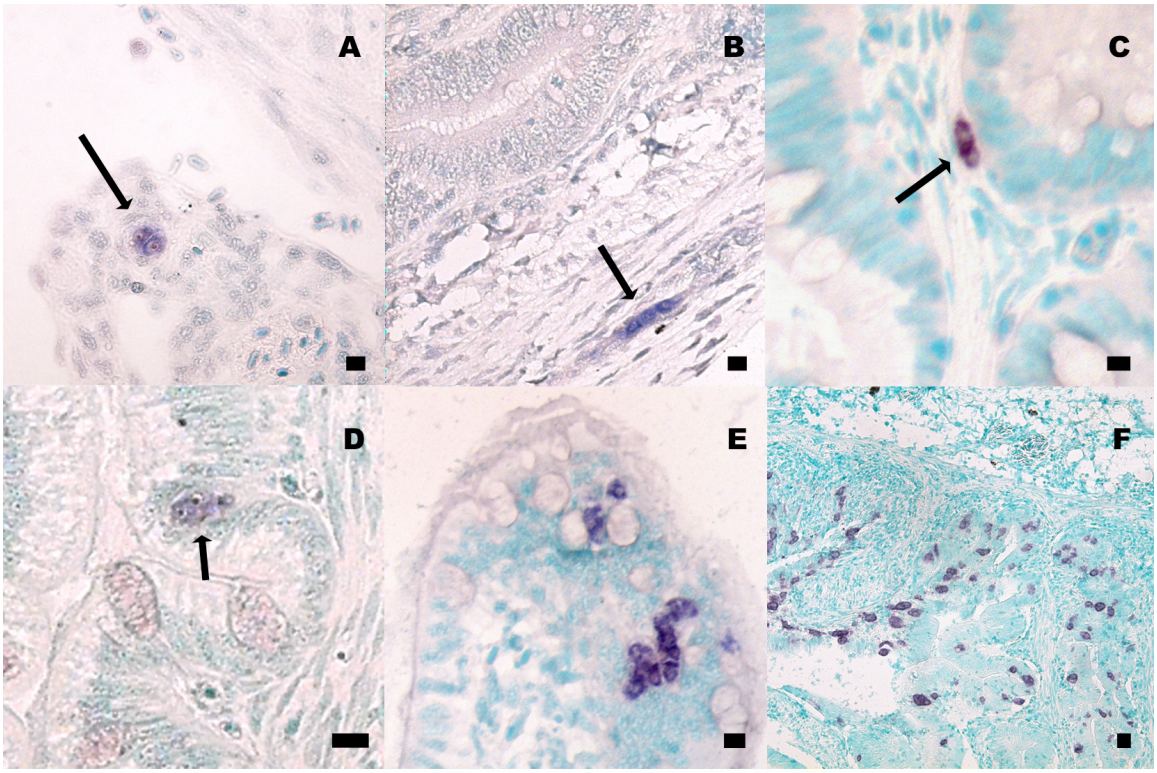

Figure 6.4. *Ceratomyxa shasta* invasion and proliferation in the intestine. In situ hybridization, scale bars 10 µm. A) Blood stages of the parasite (arrow) in blood vessels surrounding the intestine, 5 days p.i. B) Parasites (arrow) migrating through the serosa. C) Parasites (arrow) migrating through the lamina propria, 5 days p.i. D) Early parasite proliferation (arrow) at the interface between lamina propria and epithelium, 5 days p.i. E) Parasite proliferation 1 week p.i. F) Parasite proliferation in all layers of the intestine and resulting inflammation and tissue destruction (2 weeks p.i.).

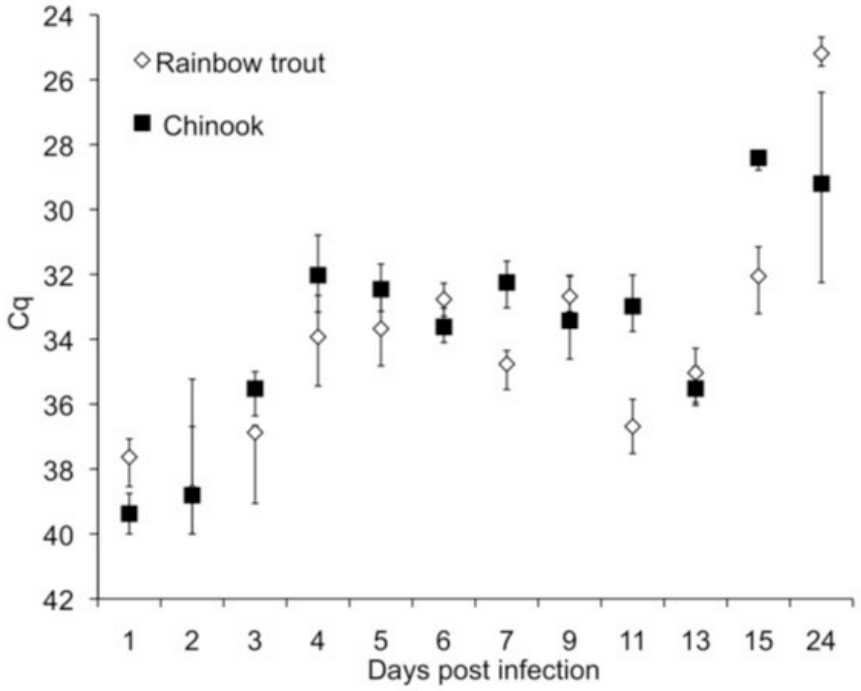

Figure 6.5. Relative quantity of parasite DNA in the blood of rainbow trout and Chinook salmon after infection with *Ceratomyxa shasta*. Error bars indicate the range of quantitative cycle (Cq) values obtained for the two fish sampled each day after exposure.

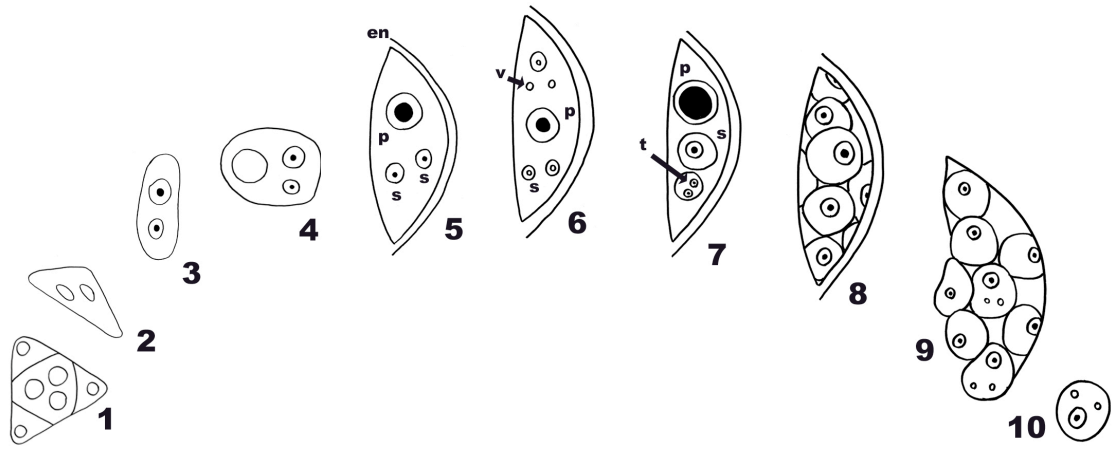

Figure 6.6. Proposed pre-sporogonic development of *Ceratomyxa shasta* in the fish host. 1. Actinospore. 2. Binucleate sporoplasm released from actinospore to the surface of the gill epithelium. 3. Binucleate sporoplasm in gill epithelium. 4. Developing parasite before autogamy of nuclei in gill arch blood vessel. 5. Parasite with primary nucleolus and secondary nuclei (s) enclosed beneath endothelium (en). 6. Primary cell developing up to eight secondary cells (s), primary nucleus (p), and vegetative nuclei (v) in cytoplasm of primary cell. 7. Secondary cells enlarge (s) and differentiate with the development of tertiary nuclei (t), primary nucleus (p) enlarges. 8. Primary nucleus has dissolved, eight mature secondary cells (s) remain enclosed in a primary cell beneath endothelium. 9. Primary cell containing mature secondary cells ruptures and secondary cells released into blood stream. 10. Secondary cells free in blood stream, may re-invade endothelium of blood vessel and produce new primary cells with secondary cells or migrate to intestinal epithelium.

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ACKNOWLEDGEMENTS

The authors sincerely thank Adam Ray and Rich Holt for their assistance with field exposures as well as Fisher's RV Park for land access to our field site. Additionally, we thank Don Stevens, Harriet Lorz and Charlene Hurst for help in maintaining polychaetes in the lab as with fish care and sampling; Gerri Buckles for qPCR assay, Dolly Baxa of the Hedrick laboratory at the University California, Davis for advice in optimizing the *in situ* hybridization protocol, and Sascha Hallett for reviewing the manuscript.

CHAPTER 7: COMPARISON OF HOST RESPONSE TO *CERATOMYXA SHASTA* INFECTION IN SUSCEPTIBLE AND RESISTANT STRAINS OF CHINOOK SALMON

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For submission to Fish and Shellfish Immunology

ABSTRACT

Variations in resistance to ceratomyxosis, an intestinal disease caused by the myxozoan parasite, *Ceratomyxa shasta*, have been reported between strains of Chinook salmon, *Oncorhynchus tshawytscha*. It is hypothesized that resistant fish prevent parasite invasion and/or prevent parasite establishment in the blood and intestine as well as mount a more effective immune response. To test these hypotheses, the progress of *C. shasta* infection in high susceptibility Salmon River Chinook salmon (SR) was compared to that of the resistant Iron Gate strain (IG). Comparison of invasion and early infection in the gills showed no differences in infection intensity or location of the parasite between strains, indicating that resistance does not occur at these early stages of establishment and replication. However, DNA assay of blood demonstrated that the resistant fish eliminated the parasite from the blood 2 wks after infection when challenged with a sub-lethal parasite dose. Histological examination of tissues showed that parasites in the intestines of these fish were either isolated in foci of inflammation or migrated into the intestinal lumen. Both strains up-regulated the expression of IL-10 and IFNγ in the blood and IL-6, IL-8, IL-10 and IFNγ in the intestine in response to infection on day 12. All of the susceptible strain succumbed to infection within 24 days whereas all of the resistant strain survived. IL-10 and IFNγ expression increased in the blood of resistant fish on day 25. IFNγ expression increased further in the intestine in the resistant strain on day 25 but the expression of IL-6, IL-8 and IL-10 remained the same. By day 90 cytokine expression returned to control levels and resistant fish had recovered. Based on the cytokine expression and histology, the inflammatory response of the susceptible strain was delayed and incapable of containing or eliminating *C. shasta.* Thus, it appears that resistant fish: 1) limit the number of parasites that reach the intestine by clearance from the blood stream; 2) elicit a rapid and effective inflammatory response that both traps and lyses *C. shasta* early on and 3) develop tolerance to the parasite in which parasite proliferation is limited, damage to host tissue is prevented and ultimately the parasite is expelled into the lumen prior to maturation.

Keywords: *Ceratomyxa shasta*, Chinook salmon, resistance, immune response, cytokine, parasite

INTRODUCTION

Variations in resistance to ceratomyxosis, an intestinal disease caused by the myxozoan parasite, *Ceratomyxa shasta*, have been reported between strains of Chinook salmon, *Oncorhynchus tshawytscha*. Salmonid strains that migrate through areas enzootic for *C. shasta* have increased resistance to this parasite (Ching and Munday, 1984; Hemmingsen et al., 1986; Ching and Parker, 1989; Ibarra et al., 1991; Ibarra et al., 1992; Bartholomew, 1998; Bartholomew et al., 2001). Resistance to *C. shasta* is an inherited trait (Ibarra et al., 1992, 1994; Bartholomew et al., 2001) conferred through unique genetic loci (Nichols et al., 2003) but the mechanism is unknown. Although these strains are considered "resistant" they may become infected and succumb to disease when under conditions of high water temperature (Udey et al., 1975) or high parasite dose (Ratliff, 1983; Stocking et al., 2006). Previous researchers propose the involvement of two strategies in defense against *C. shasta*: resistance of parasite entry and establishment, and the mounting of an effective immune response against parasites that evaded the primary defenses (Johnson, 1975; Ratliff, 1981; Bartholomew et al., 1989b; Ibarra et al., 1994).

Interpretation of fish host response to *C. shasta* infection from previous studies is complicated by varying parasite dose. While a single parasite may cause lethal infections in a susceptible strain of fish (Bjork and Bartholomew, 2009), the infection threshold for a resistant strain was estimated to be as low as 56,000 actinospores per fish (R. Adam Ray, personal communication). However, once this dose is delivered, the course of ceratomyxosis is similar in susceptible and resistant strains (Bjork and Bartholomew, 2009; Bjork and Bartholomew, submitted). In lethal infections, the parasite invades the gills and enters the bloodstream, which serves both as a site of proliferation and means of transport to the intestine. In the intestine, parasites proliferate between mucosal epithelial cells of the intestine and lymphocytes infiltrate throughout the intestinal tissue (Bartholomew et al., 1989b; Bjork and Bartholomew, submitted). In resistant Chinook salmon that received lethal doses of the parasite, there was no evidence of parasite exclusion or containment in the gills or in the intestine. In another study using this same Chinook salmon strain, plasma lysozyme, complement and phagocytosis were increased after challenge, but again the

infecting dose was lethal and these innate defenses failed to provide protection (Foott et al., 2004). In contrast, in resistant fish infected with sub-lethal doses of the parasite, parasite was contained within granulomata (Ibarra et al., 1991; Foott et al., 2004) or parasites were detected in the lumen of the intestine, presumably after migrating through the lamina propria and epithelium (Bartholomew et al., 1989b).

The goal of this study is to determine the defense strategies resistant fish developed to survive *C. shasta* infection. To determine the location and timing of parasite containment or elimination in the resistant fish, the pathology of infection in resistant and susceptible strains was compared. The expression of pro-inflammatory (IL-1β, IL-2, TNFα, IL-6, IL-8, and IFNγ) and regulatory (IL-10, and TGF β) cytokines in blood and intestine in response to *C. shasta* was compared to identify differences in the regulation of the immune response between the strains.

MATERIALS AND METHODS

Fish stocks: A coastal strain of Chinook salmon from the Salmon River (SR) Hatchery, OR was selected as the susceptible strain. Klamath River Chinook salmon from Iron Gate (IG) Hatchery, CA were selected as the resistant strain (Foott et al., 2004; Stocking et al., 2006). All fish were transferred to the John L. Fryer Salmon Disease Laboratory (SDL), Oregon State University, Corvallis, OR, fed a daily commercial diet (BioOregon) and reared in 13°C specific pathogen free (SPF) well water until initiation of the experiment, except for fish in Experiment II which were transferred directly from the hatchery to the field site.

Experiment I. Resistance at the portal of entry (gills): To compare *C. shasta* actinospore invasion in the gills of SR and IG, 10 fish of each strain were challenged for 24 h to an estimated parasite dose of 4,300 actinospores/fish by exposure in the outflow of aquaria containing infected polychaetes. As a source of actinospores, a culture of *M. speciosa* was infected with myxospores obtained from Chinook salmon using methods previously described (Bjork and Bartholomew, 2009). Three 1L water samples were collected at the beginning of the challenge and assayed by quantitative PCR (qPCR) (Hallett and Bartholomew, 2006). Water flow rate through the tanks and spore concentration from qPCR was used to estimate the parasite dose.

After 24 h, 5 fish from each strain were euthanized with an overdose of MS222 (tricainemethosulfonate, Argent) and the gills from one side of the head from each fish was fixed for histology in Davidson's fixative for 24 h then transferred to 70% ethanol. The other set of gills was weighed and frozen for qPCR. The remaining 5 fish from each strain were transferred to 13°C SPF well water flow through tanks to monitor for the development of infection for 60 days. As a control, 5 uninfected fish from each stock were maintained identically. Because of the limited availability of these fish, gills were not collected for qPCR from control fish at 24 h; they were assessed for the presence of *C. shasta* infection in the intestine by PCR (Palenzuela et al., 1999) at termination of the experiment.

DNA extraction of gill tissue was done using the Qiagen DNeasy Blood and Tissue kit. DNA was eluted in 60 µl, re-applied to the column and eluted again to increase yield. The DNA was then analyzed by qPCR (Hallett and Bartholomew, 2006). Each sample was run twice in the qPCR assay and the Ct values were averaged for each sample. Two internal positive controls (DNA from an infected fish tissue and an artificial parasite DNA template) and a negative control (molecular grade water) were included. If the standard deviation of the replicates was greater than 1, the samples were re-run. Samples for which parasite DNA was not detected were assigned a Cq of 40 to facilitate data analysis. A student's t-test was performed to determine if there was a significant difference in the Cq values between stocks. S Plus statistical software 8.0 (Insightful Technologies, Tibco Software, Palo Alto, CA) was used for statistical analysis.

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

Experiment II. Location of parasite containment and elimination: Resistant IG Chinook salmon (15.4 g ± 3.1) were exposed to a sub-lethal parasite dose by holding held in cages in the mainstem of the Klamath River, river mile 259.1, above Beaver Creek, CA (Stocking et al., 2006) for 24 h in September 2008. After exposure, fish were transported to the SDL and reared on 18°C SPF well water in 25 L flow through

tanks. Fish were treated with furanase (Aquarium Products, Glen Burnie, MD) and fed antibiotic-medicated food to minimize bacterial infections. Using data from water samples and flow measured during exposure, the exposure dose was estimated to be 165,000 parasites per fish (R. Adam Ray personal communication).

After the 24h exposure (Day 1) 2 fish were euthanized with an overdose of buffered MS222 and blood samples (between 10 and 30 µL were collected and frozen, the fish were then fixed in Davidson's fixative for 24 h then transferred to 70% ethanol. Two fish were collected daily for 2 weeks following exposure, then every 3 days for 2 weeks; thereafter sampling was done bi-weekly. An additional group of non-exposed fish was included as controls. From the fixed fish, gills, stomach, kidney, heart, brain, spleen, liver, pyloric caeca, intestine, and samples of skin muscle and fins were dissected and processed by routine histological methods, embedded in paraffin and serial 5 µm sections were adhered to Superfrost plus slides for staining.

Parasites in tissue sections were labeled using *in situ* hybridization protocols developed by Palenzuela and Bartholomew (2002) and modified by Bjork and Bartholomew (submitted). Alternate sections were stained with May-Grunwald Giemsa or Mayer's H & E. DNA from blood was prepared and analyzed by qPCR as described by Bjork and Bartholomew (submitted).

Experiment III. Comparison of cytokine expression: IG and SR strains were challenged at the same parasite dose at the SDL using the same infected polychaete colony as described for the first experiment. 30 IG and SR Chinook were each placed in a 40 cm cylindrical cage with 15 cm diameter and held in a 30 gal flow through aquarium receiving water from the outflow of the infected polychaete colony for 24 h. An equal number of fish from each strain was held in cages in UV treated Willamette River water at the same flow rate for 24 h as a negative control to mimic the water quality parameters that the infected fish experienced. After exposure, each treatment group (IG infected, IG control, SR infected, SR control) was divided equally into three 25L SPF flow through tanks at 13°C (a total of 12 tanks, 3 for each treatment group).

Nine fish from each treatment group (3 per replicate tank) were sampled 12, 25 and 90 days after infection. Fish were euthanized with an overdose of MS222 and 60 µl of blood was collected from the caudal vein. A Qiagen Blood RNA extraction kit was used to lyse and remove the red blood cells; WBCs were isolated, lysed and frozen at -80 ºC until RNA extraction per kit instructions. A section of the posterior intestine (30 mg, approximately 0.5 cm) was excised, preserved in RNAlater and stored at -80ºC until RNA extraction. The remainder of the intestine of each fish was preserved in Davidson's fixative and processed for histology as previously described. Alternate section sections were stained with Giemsa or H & E. and examined for presence of the parasite and associated pathology.

Parasite intensities and number of inflammatory foci were determined by examing the histological sections at 20 x and assigning a score based on the following criteria. Parasite intensity was scored on a scale of 0-5. Sections in which no parasites were observed were scored as 0, sections with 1-5 foci of parasites were scored as 1, 6-10 foci of parasites were scored as 2, 11-15 foci of parasites were scored 3, 16-20 foci of parasites were scored as 4 and sections having greater than 20 parasites were scored 5. Similarly the number of inflammatory foci was scored, areas of confluent inflammation were estimated to be a number of foci based on the number of villi over which the inflammation encompassed. For scoring of inflammation, a score of 0 was assigned to sections in which no inflammation was observed, 1-10 inflammatory foci was scored 1, 11-20 scored 2, 21-30 scored 3, and greater than 30 scored 4. T-tests were used to compare infection intensity and inflammation between SR and IG fish at day 12 and between IG at the later sampling times.

RNA isolation from blood proceeded as per kit instructions. Intestinal tissue from individual fish was removed from RNA later and a 30 mg piece was removed and the RNA extracted using RNeasy Minikit with in column DNAse I treatment (Qiagen, Inc). RNA concentration and purity was determined by Nanodrop and RNA samples were either diluted or concentrated using a Savant speed vac concentrator with refrigerated vapor trap prior to cDNA synthesis.

To synthesize cDNA, 500 ng oligo dT primers, 10 mM dNTPs, and 4µg of RNA from the intestine or 1 µg of RNA from blood from each fish, were incubated at

65ºC for 5 min then placed on ice for 1 min. 10x RT buffer, 25mM $MgCl_2$, 0.1M DTT and 10 U RNase OUT (RNase inhibitor) were added and incubated at 42ºC for 2 min, followed by the addition of 50 units Invitrogen Superscript III. Incubation continued for an additional 50 min at 42ºC, proceeded by 10 min at 70ºC then held at 4ºC. RNase H (40 U) was added and the samples were incubated at 37ºC for 20 min. All reagents for cDNA synthesis were obtained from Invitrogen Life Technologies (Carlsbad, CA, USA).

Primers for amplification of IL-1β, IFNγ, TNFα, and IL-8 were designed from sequences from Chinook salmon in GenBank. Primers for amplification of IL-2, IL-6, IL-10, and TGF β transcripts were designed based on rainbow trout sequences in GenBank. Serial dilutions of a standard cDNA preparation were used to assess PCR efficiency. Primer concentrations and reaction efficiency and GenBank accession numbers are listed in Table 7.1. The dissociation curve of the PCR products were analyzed to verify a single peak and products were separated by gel electrophoresis to corroborate the presence of only one product and to evaluate product size.

Quantitative PCR was performed in 10µl reactions containing 5 µl Power SYBR Green Master Mix (Applied Biosystems, Foster City, CA) 1 µl of forward and reverse primer and 2 µl cDNA and 2µl of cDNA. For each gene, 1:10 dilution of cDNA from intestine of each sample was used and 1:5 dilution of cDNA from blood was used due to a lower RNA yield from blood samples. A β actin PCR product was synthesized and included in each assay as an endogenous control and to assess interassay variation. MicroAmp Fast Optical 96 well reaction plates were used. Each sample was run in duplicate wells through 40 cycles on an ABI 7500 Fast Real-time PCR system on the Standard 7500 setting with an added dissociation step and ROX used as passive reference. After 50ºC for 2 min, amplification proceeded for 40 cycles at: 95ºC for 10 min, 95ºC for 15 s, 60ºC 1 min, then the dissociation curve consisted of 95ºC for 15 s, 60ºC for 1 min followed by 95ºC for 15 s. Only samples for which both wells fluoresced were considered positive. If the standard deviation of the duplicate wells for an individual sample was >1, the sample was re-run.

The comparative C_T method was used and results are reported as relative transcription to n-fold difference in a calibrator cDNA (User Bulletin #2 Applied

Biosystems, Eder et al 2009). β-actin was the endogenous control and used to normalize target gene expression (ΔC_T) and the calibrator was the lowest expression of the target gene in control fish. The ΔC_T for each experimental sample was subtracted from the ΔC_T of the calibrator, therefore the linear amount of target molecules relative to the calibrator was calculated by $2^{-\Delta\Delta C_T}$. Therefore, all cytokine gene transcription is expressed as an n-fold difference relative to the calibrator.

Initially, the expression of all cytokines was measured from 3 pools of 3 fish each (1 fish from each replicate tank per pool) from the infected and control fish for each strain. When the difference between the infected and control fish were significantly different, the expression from individual fish was measured. T-tests were used to determine significant differences ($p < 0.05$) between fold differences relative to the calibrator of infected and control fish within a strain, and data was log transformed to meet the assumptions of the statistical tests. S Plus 8.0 statistical software (Insightful Corp, TIBCO Software Inc.) was used for statistical analysis. To determine if the log fold expression of each of the cytokines was significantly different between infected and control IG over time, we tested for differences using multivariate analysis of variance (MANOVA). If the MANOVA was significant (Wilks' $\lambda < 0.05$), we used individual ANOVAs and Tukey's Honestly Significant Difference (HSD) tests to interpret results ($p<0.05$). To test for differences between IG infected and control and SR infected and control on day 12 one way ANOVA was used followed by Tukey's HSD tests.

RESULTS

Experiment I. Resistance at the portal of entry (gills): Both IG and SR Chinook salmon gills tested positive by qPCR. The Cq values between the two strains were not significantly different (t-test $p=0.56$) and averaged 29.97 ± 1.7 for SR and 30.46 ± 0.77 for IG. Histology revealed no differences in the parasite numbers in the gills and parasite detection by histology was low overall. All of the SR Chinook salmon held after the exposure succumbed to infection, with a mean day to death of 32 (± 9). All of the IG Chinook salmon survived and myxospores were not detected upon termination at 60 days as determined by wet mount examination. All of the control fish were negative by PCR.

Experiment II. Location of parasite containment and elimination: *In situ* hybridized histological sections of IG strain Chinook salmon exposed to a sub-lethal parasite dose show *C. shasta* in the gill filament epithelium and blood vessels during the first three days after exposure. At 7-10 d, parasites are found in the serosa of the intestine, lamina propria and intestinal epithelium (Figure 7.1 A-C). At 2-3 wks, foci of granulomatous inflammation are present, primarily in the serosa and sub-mucosa of the intestine, and trophozoites are seen within these foci 2 wk p.i. (Figure 7.1 D, E). At 3 wks p.i. trophozoites were present only in the lumen of the intestine (Figure 7.1 F). At 5 wks, a few foci of inflammation were observed in the intestine and one of the two fish had inflammation in the lamina propria, however, no parasites were identified in these foci. At 7 wks, one fish showed minor inflammation in the lamina propria. *Ceratomyxa shasta* was not detected in the liver, pyloric caeca, pancreas or kidney at any of the sampling times. However, vascular congestion on the surface of the spleen was noted at 2 and 3 wks p.i.

Ceratomyxa shasta DNA was detected in the blood of sub-lethally infected fish by qPCR (Figure 7.3) at 4 d p.i., when the Cq was 35.9 (average of 2 fish). Cq values ranged between 34.2 and 36.7 until d 13 p.i., thereafter the average Cq was 40, with a range of 36.28 to undetectable until the end of the experiment. The NTC was undetected in all plates, and the Cq value of the positive controls had a standard deviation of 0.40 for tissue and 1.36 for the artificial template between qPCR plates. Only samples for which both wells fluoresced were considered positive for parasite DNA.

Experiment III. Comparison of regulation of immune response: All of the IG strain challenged survived to 90 d p.i. Infection in the SR strain was fatal (mean day to death 22 d ± 0.6), thus they were sampled only at 12 d p.i. Therefore, only IG infected and control fish are compared at 25 and 90 d.

On day 12, *C. shasta* trophozoites were observed in all layers of the posterior intestine (mucosal epithelium, lamina propria, muscularis, and serosa) of all of the 9 SR infected fish, but most prominently in the mucosal epithelium (Figure 7.2 A). SR infected fish exhibited mild to moderate multi-focal and locally extensive inflammation in the lamina propria dominated by mononuclear cells and a few

neutrophils (Figure 7.2 B). Parasite intensity in the SR fich ranged from 11 to 49 foci of parasite proliferation and scored between 3 and 5 (Figure 7.6) and is significantly different than IG on day 12 ($p < 0.001$). Less than 5 foci of parasites (on average) were detected in IG infected fish on day 12 and these parasites were in the serosa or within the lamina propria. Although parasites were not noted in all IG infected fish, fish exhibited moderate to diffuse lymphocytic enteritis and the mean inflammation score of these fish was 2.7 as compared to 1.6 for SR (Figure 7.6) and this difference was significantly different ($p = 0.004$). In 3 fish this mononuclear cell response was transmural and scattered macrophages were present (Figure 7.2 C). Two of these fish exhibited increased vascularization in the lamina propria, evidenced by dilated blood vessels. *Ceratomyxa shasta* was not detected in any of the control fish and none showed any signs of inflammation in histology.

On day 25, *C. shasta* trophozoites were detected in 5 IG infected fish (Figure 7.6). Inflammation was predominantly in the lamina propria and in the mesentery adipose. One fish was heavily infected with the parasite and scored a 5, whereas the other fish were 0 to1. Inflammation of mononuclear cells was largely limited to the lamina propria with some fusion of the villi and trophozoites were seen among the infiltrated cells in at least 2 fish. Inflammation in another 2 fish was more diffuse, extending through the muscularis and serosal surface into the mesentery adipose. In one of these fish, parasites were contained within the mesentery adipose but numerous trophozoites were also present in the epithelium and destruction of villi was evident. In another fish, the steatitis extended from the serosal surface and surrounded a blood vessel (Figure 7.2 D). On day 90, parasites were found in the lumen of the intestine of only 1 fish, whereas no parasites were observed in the other 8. Inflammation in the lamina propria was minimal (Figure 7.6) or absent and in one fish, lymphocytes were scattered in the mesentery adipose.

There was not a significant difference between parasite intensity scores in IG from day 12 to 25 ($p = 0.84$) or from day 25 to day 90 ($p = 0.19$), but the difference between day 12 and day 90 was significant ($p = 0.016$). Inflammation scores in IG also changed over time, where day 12 to 25 was not significant ($p = 0.6$) but day 25 to

day 90 ($p < 0.001$) and day 12 compared to day 90 ($p< 0.001$) was also significantly different.

Four SR control blood samples were damaged during processing and were eliminated from the analysis on day 12 and 2 IG control blood samples were eliminated from day 25 samples due to insufficient RNA. The blood of both infected IG and SR infected fish showed significant increases in IL-10 (SR $p=0.014$, IG $p<0.001$) and IFNγ (SR $p<0.001$, IG $p= 0.003$) on day 12 p.i. compared to their respective controls (Figure 7.4). The average log fold change in expression of IFNγ was approximately 1.5 times higher in SR infected fish compared to the control, and IL-10 expression was nearly twice as high (Table 7.2). In the IG strain the difference between infected and control fish was much lower, with the average expression of IL-10 and IFNγ in infected fish twice as high as in the control. On day 25, the average log fold expression of both IFNγ and IL-10 was at least twice as high in IG infected than in controls, however, the infected group had high variability and the differences in IFNγ were not significantly different (Figure 7.4, Table 7.2). The average log fold expression of both IFNγ and IL-10 on day 90 was not significantly different between infected and control IG Chinook salmon. MANOVA followed by Tukey's HSD identified significant differences in the expression of both cytokines over time (Table 7.2). There were no significant differences in the expression of IL-1β, IL-4, IL-6, IL-8, TNFα or TGFβ at any sampling time.

In the intestine on day 12 there were significant increases in infected SR in comparison with the controls in the transcription of IL-6 ($p <0.001$), IL-8 ($p = 0.006$), IL-10 ($p < 0.001$) and IFNγ (<0.001) (Figure 7.5). Although the expression of these cytokines in infected IG was almost double the expression of IG controls, expression was variable and only the difference in IFNγ was significant ($p = 0.02$). On day 25, only the expression of IFNγ was significantly different between IG infected and control, and log fold change in expression was more than twice as high in infected fish (Figure 7.5). There were no significant differences in the expression of IL-1β, IL-4, TGFβ or TNFα on day 12 or 25. On day 90, all cytokine expression in both of the groups decreased and the expression of IG infected and control were not significantly different at this time.

DISCUSSION

In this study, the host response of resistant fish to *C. shasta* infection was followed from the site of invasion to parasite clearance and resolution of the infection in the intestine. Four hypotheses surrounding fish host resistance to *C. shasta* were investigated: resistance of parasite invasion into the gills, elimination of parasite from the blood prior to arrival in the intestine, prevention of establishment/proliferation in the intestine, and the activation of an effective immune response. We found no differences in parasite penetration of the gills between resistant IG and susceptible SR Chinook salmon, nor were there differences in the migration of *C. shasta* into the blood vessels. Thus there is no evidence to support resistance to invasion in the gills. However, when IG Chinook salmon were infected with a sub-lethal dose, this resistant strain eliminated the parasite from the blood 2 weeks p.i., and fewer parasites arrived in the intestine as compared to the susceptible strain. At this point, two host defense strategies were observed in the intestine. In one strategy, parasites were isolated in foci of inflammation in the intestine 2-3 wks p.i. and eliminated. Alternatively starting 3 weeks p.i.; trophozoites were present in the lumen of the intestine without diffuse parasite proliferation or destruction of host tissue, and is indicative of parasite tolerance.

This study was the first to examine the cytokine expression in Chinook salmon in response to *C. shasta* infection and differences in the expression of pro-inflammatory and regulatory cytokines between susceptible and resistant fish were identified. In the blood IL-10 and IFNγ were significantly up-regulated in both strains of fish. The parasite replicates in the endothelium of blood vessels, thus up-regulation of IFNγ in the blood could indicate a recruitment of lymphocytes to these sites or it could be a snapshot of the expression of cells in the blood coordinating the response to the infection in the intestine. The expression of IL-10 in the blood of susceptible fish was higher than in resistant fish at any time sampled. IL-10 deactivates activated immune responses but is also associated with mast cells/eosinophilic granular cells (EGCs) (Thompsonsnipes et al., 1991). EGCs are associated with the response of both brown trout and the resistant strain of Hofer rainbow trout to the myxozoan *Myxobolus cerebralis* (Hedrick et al., 1998; Baerwald et al., 2008). Hence, the

increase in IL-10 in the blood could indicate a role for EGCs in eliminating blood stages of *C. shasta* or may simply reflect the host's attempt to regulate inflammation occurring in the intestine and/or in response to parasite proliferation in the endothelium of blood vessels.

IL-6, IL-8, and IFNγ are all pro-inflammatory cytokines and their expression in the intestine in addition to expression of the regulatory cytokine IL-10 coincided with inflammation in the intestine in both strains of fish. IL-6, which is involved in T-cell differentiation and the acute phase response in liver (Hirano, 1992; Secombes et al., 2009), is up-regulated to a similar extent in both strains of fish and indicates an activation of T-cells. IL-8 is a CXC chemokine involved in the recruitment of neutrophils (Laing and Secombes, 2004), which are present during inflammation in the intestine but are not the dominant inflammatory cell in either strain. Thus, the nearly equivalent expression of IL-8 between the two strains is not surprising. The expression of IFNγ, which is involved in the recruiting cells to the site of infection, increasing antigen presentation, and enhancement of NK cell activity, as well as the induction of lytic pathways such as nitric oxide synthase (NOS) and phagocyte oxidase (Randelli et al., 2008); is increased in response to infection in both strains but expression in the susceptible strain was higher. Inflammation in susceptible fish occurred only in the lamina propria and did not prevent parasites from reaching the epithelium or arrest proliferation; whereas lymphocytic infiltration into the intestine of resistant fish is transmural in some foci and parasites are limited to the serosa or lamina propria. The high IFN expression in susceptible fish and lower intensity of inflammation indicates that the Th1 response in this strain was delayed in comparison to resistant fish. Significant differences were not detected in IL-1β, IL-2, TNFα, or TGFβ, however examination of other time points or tissues may reveal differences in the expression of these cytokines between the strains.

Only the resistant strain of fish survived past 24 days and there was a progressive resolution of the inflammation in the intestine by 90 days p.i. At day 25 p.i., IL-10 and IFNγ expression in the blood increased, but the expression of IL-6, IL-10, and IFNγ in the intestine was nearly equivalent to day 12. Trophozoites were found in the intestinal lumen of resistant fish during the latter stages of sub-lethal

infections, but little to no inflammation was observed in the intestine of these fish. This is suggestive of parasite tolerance which is an adaptive response and indicates the switch to Th2 response. Monitoring the expression of IL-12 or IL-4, which more specifically regulate Th1 or Th2 respectively (Jankovic et al., 2001), as well as expression analysis in the head kidney and spleen, could provide more information on the differentiation of this response and possibly differences in its regulation between susceptible and resistant fish.

The Th2 response is the expected adaptive immune response to extracellular parasites, and the development of an antibody response to *C. shasta* in a susceptible strain of rainbow trout has been demonstrated. Rainbow trout that survive *C. shasta* infection produce both serum and mucosal antibodies against the parasite, and mucosal antibody in the lamina propria coincides with parasites in the lumen of the intestine of these fish (J. Oriol Sunyer, personal communication). These observations in susceptible fish and the observed translocation of immature *C. shasta* parasites into the intestinal lumen of resistant fish in the current and previous studies (Bartholomew et al., 1989a; Ibarra et al., 1994; Bartholomew et al., 2004b) provides a compelling link between parasite expulsion into the lumen of the intestine, development of an antibody response, and parasite tolerance. Given the similarities within *Oncorhynchus* spp., and the higher resistance to and recovery from infection of the IG Chinook salmon strain used in the present study, an antibody response to *C. shasta* is anticipated and should be investigated in the future. An antibody response could offer protection to the fish during subsequent encounters with the parasite as has been demonstrated for other myxozoans (Griffin and Davis, 1978; Ryce, 2003; Sitjà-Bobadilla et al., 2007).

The more rapid and effective elimination of the parasite in the resistant fish could be facilitated through a variety of inherited immune traits. More efficient host defenses could be due to an inherently greater abundance of immune cells and a better primed immune response overall, but this is not supported by other studies in which fish that are resistant to a given myxozoan remain susceptible to other pathogens (Hedrick et al., 2001; Bartholomew et al., 2004a; El-Matbouli et al., 2009). Other traits, such as pattern recognition receptors (PRRs) are vertically transmitted

(Magnadottir, 2006), and PRRs specific to *C. shasta*, would prompt earlier phagocytosis or lysis of the parasite and quicker activation of other components of the immune response. Resistant fish strains may also have unique complement isoforms that recognize *C. shasta* antigens better than susceptible strains. Jankovic et al. (2001) hypothesize that innate/genetic factors dictate the T-cell receptor (TCR) affinities on antigen presenting cells, which in turn affects the activation of either Th1 or Th2 response. Differences in TCR affinity between the strains of Chinook analyzed in this study may have been responsible for the earlier inflammation in the intestine by activation of the Th1 in resistant fish as well as the differentiation of the Th2 response which led to resolution of the inflammation and parasite tolerance. Qualitative differences in TCR affinity have been demonstrated in hosts with varying susceptibility to *Leishmania major,* with resistant hosts having high affinity and susceptible hosts low affinity TCRs (Malherbe et al., 2000). Optimum allelic diversity of the major histocompatibility complex (MHC) is associated with differences in the resistance and innate immune response of three spine stickle backs to parasites (Kurtz et al., 2004) and should be considered a candidate for driving *C. shasta* resistance as well.

Based on the observations of the current study and previous reports, *C. shasta* resistant fish: 1) limit the number of parasites that reach the intestine by clearance from the blood; 2) elicit a rapid and effective inflammatory response that both traps and lyses *C. shasta* and 3) develop tolerance to the parasite in which parasite proliferation in the intestinal epithelium is limited, damage to host tissue is prevented and ultimately the parasite is expelled into the lumen. The success of this response is dependent on parasite dose and temperature. In our study, resistant Chinook salmon infected with as few as 4,300 and as many as 165,000 actinospores per fish mounted a successful immune response and recovered from infection at 18ºC by 90 days p.i. The exact mechanisms of parasite removal require further study, and future studies should further characterize the effects of parasite dose on the immune response. If an antibody response is protective, the minimum parasite dose required to elicit this response should be identified, as well as the maximum dose that an immune fish can remain protected against. These parameters would aid in predicting fish infection

severity in the natural environment and supply managers with a threshold of parasite doses in which *C. shasta* and its fish host can overlap but severity of ceratomyxosis is minimized.

Table 7.1. Primer sequences for Chinook salmon cytokine expression analysis, primer concentration, reaction efficiency, size of amplification product and GenBank accession number.

Gene	Primer sequences (5'-3')	Primer concentration and efficiency	Product (bp)	GenBank #
β actin	(F) GGACTTTGAGCAGGAGATGG	4 µM, 99.4%	186	Chinook salmon
	(R) ATGATGGAGTTGTAGGTGGTCT			FJ546418
IL-1β	(F) ACCGAGTTCAAGGACAAGGA	6 µM, 99.9%	181	Chinook salmon
	(R) CATTCATCAGGACCCAGCAC			DQ778946
IL-2	(F) TTTCCTTTTTGACGCTTTTTCTCA	4 µM, 99.6%	204	Rainbow trout
	(R) CGAGGCATTCTACTTTCACAGT			NM_001164065
IL-6	(F) CAGTTTGTGGAGGAGTTTCAGA	2 µM, 99.3%	118	Rainbow trout
	(R) TGTTGTAGTTTGAGGTGGAGCA			NM_001124657
IL-8	(F) GAGCATCAGAATGTCAGCCAG	6 µM, 97.5%	77	Chinook salmon
	(R) CTCTCAGACTCATCCCCTCAG			DQ778948
IL-10	(F) CTACGAGGCTAATGACGAGC	6 µM, 99.5%	100	Rainbow trout
	(R) GATGCTGTCCATAGCGTGAC			AB118099
IFNγ	(F) CAACATAGACAAACTGAAAGTCCA	4 µM, 99.4%	129	Chinook salmon
	(R) ACATCCAGAACCACACTCATCA			GT897806
TGFβ	(F) AGATAAATCGGAGAGTTGCTGTG	2 µM, 99.9%	275	Rainbow trout
	(R) CCTGCTCCACCTTGTGTTGT			X99303
TNFα	(F) ACCAAGAGCCAAGAGTTTGAAC	2 µM, 98.0%	154	Chinook salmon
	(R) CCACACAGCCTCCATAGCCA			DQ778945

Table 7.2 MANOVA results for comparison of cytokine gene expression in the blood and intestine of Iron Gate Chinook Salmon at 12, 25, or 90 days after infection. Degrees freedom (DF) type III sum of squares, mean square, F- statistic and p-value for treatment (infected or control), time (day) and an interaction between treatment and time.

Source	DF	Type III SS	Mean Square	F value	Pr > F
Blood IL-10					
Treatment	1	5.57	5.57	13.66	0.006
Day	2	15.75	7.87	19.3	<0.0001
Treatment*Day	2	3.28	1.64	4.02	0.025
Blood IFNγ					
Treatment	1	1.54	1.54	2.28	0.1383
Day	2	28.27	14.13	20.88	<0.0001
Treatment*Day	2	7.15	3.57	5.28	0.0087
Intestine IL-6					
Treatment	1	7.95	7.95	5.56	0.0225
Day	2	7.33	3.66	2.56	0.087
Treatment*Day	2	6.66	3.33	2.33	0.1086
Intestine IL-10					
Treatment	1	5.57	5.57	13.66	<0.001
Day	2	15.75	7.87	19.3	<0.001
Treatment*Day	2	3.27	1.64	4.02	0.025
Intestine IFNγ					
Treatment	1	1.54	1.54	2.27	0.138
Day	2	28.27	14.14	20.88	<0.001
Treatment*Day	2	7.15	3.57	5.28	0.0087

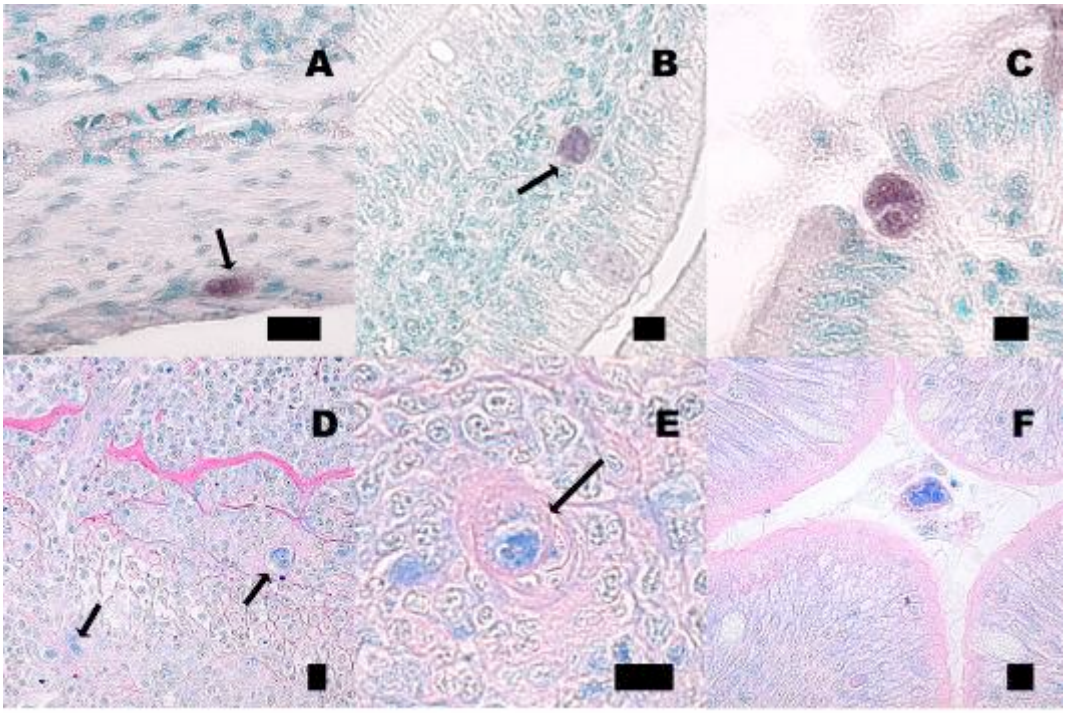

Figure 7.1. Progression of *Ceratomyxa shasta* in the intestine of sub-lethally infected Iron Gate Chinook salmon. Scale bar is 10 µm. A-C In situ hybridization, D-F May-Grunwald Giemsa stain. A. Parasite (arrow) in serosa of intestine. B. Parasite (arrow) in lamina propria. C. Parasite at epithelial surface being released into the lumen of the intestine. D. Parasite (arrows) surrounded by inflammation. E. Parasite (arrow) encased in a granuloma. F. Parasites free in the lumen of the intestine.

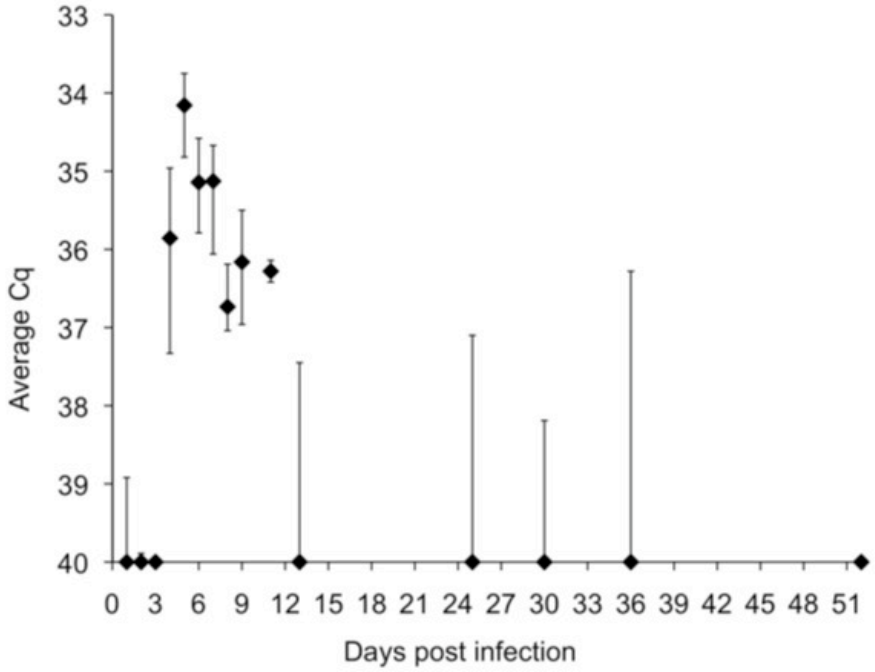

Figure 7.2. Relative abundance of *Ceratomyxa shasta* DNA in the blood of sub-lethally infected Iron Gate Chinook salmon. Error bars indicate range of Cq values for the 2 fish sampled on each day.

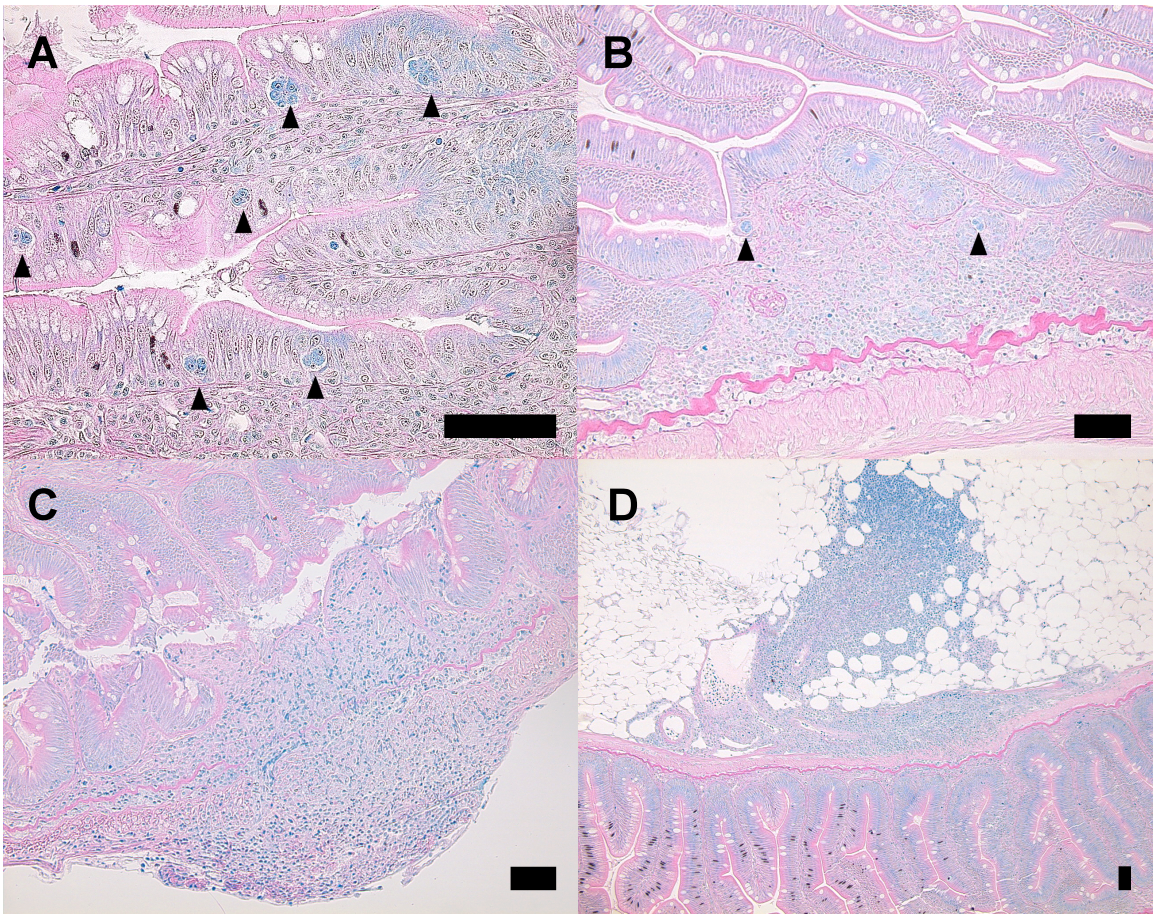

Figure 7.3. *Ceratomyxa shasta* infection intensity and inflammation in Salmon River (SR) and Iron Gate (IG) Chinook salmon. Scale bar is 50 µm. A & B. SR Chinook salmon intestine 12 days p.i. A. trophozoites (arrowheads) proliferating in intestinal epithelium (A) and inflammation in lamina propria with trophozoites (arrowheads) (B). C & D. IG Chinook salmon intestine infected with *C. shasta* C. Inflammation with lymphocyte infiltration extending from serosal surface to lamina propria in the absence of parasites. D. Lymphocytic infiltration into the mesentery adipose of IG Chinook salmon day 25 p.i. in the absence of parasites.

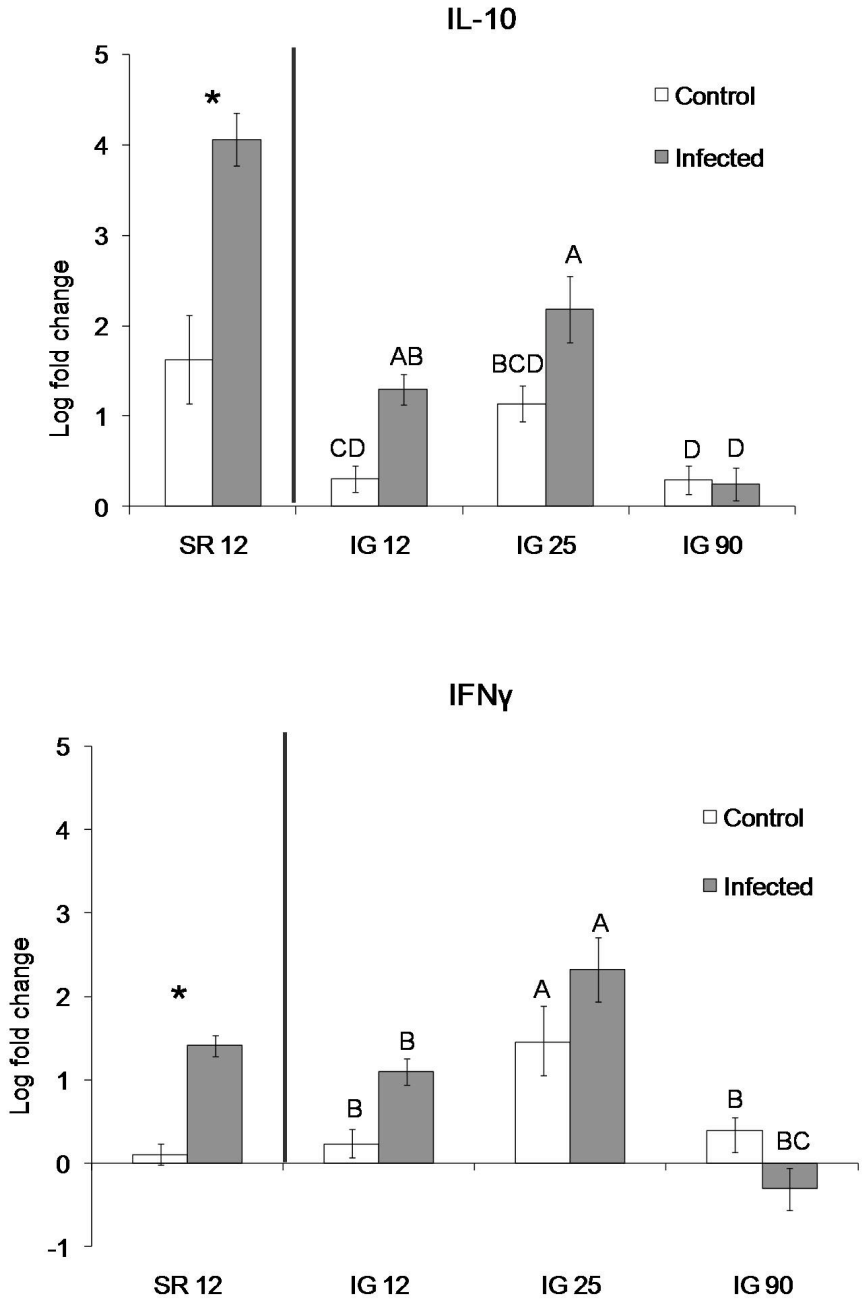

Figure 7.4. Average log fold change in IL-10 and IFNγ transcription in blood of the susceptible Salmon River strain (SR) and comparatively resistant Klamath Iron Gate (IG) strain of Chinook salmon, *Oncorhynchus tshawytscha,* after infection with *Ceratomyxa shasta*. Error bars indicate the standard error. Asterisk indicates significant difference between control and infected SR, letters indicate significant differences between IG infected and control over time.

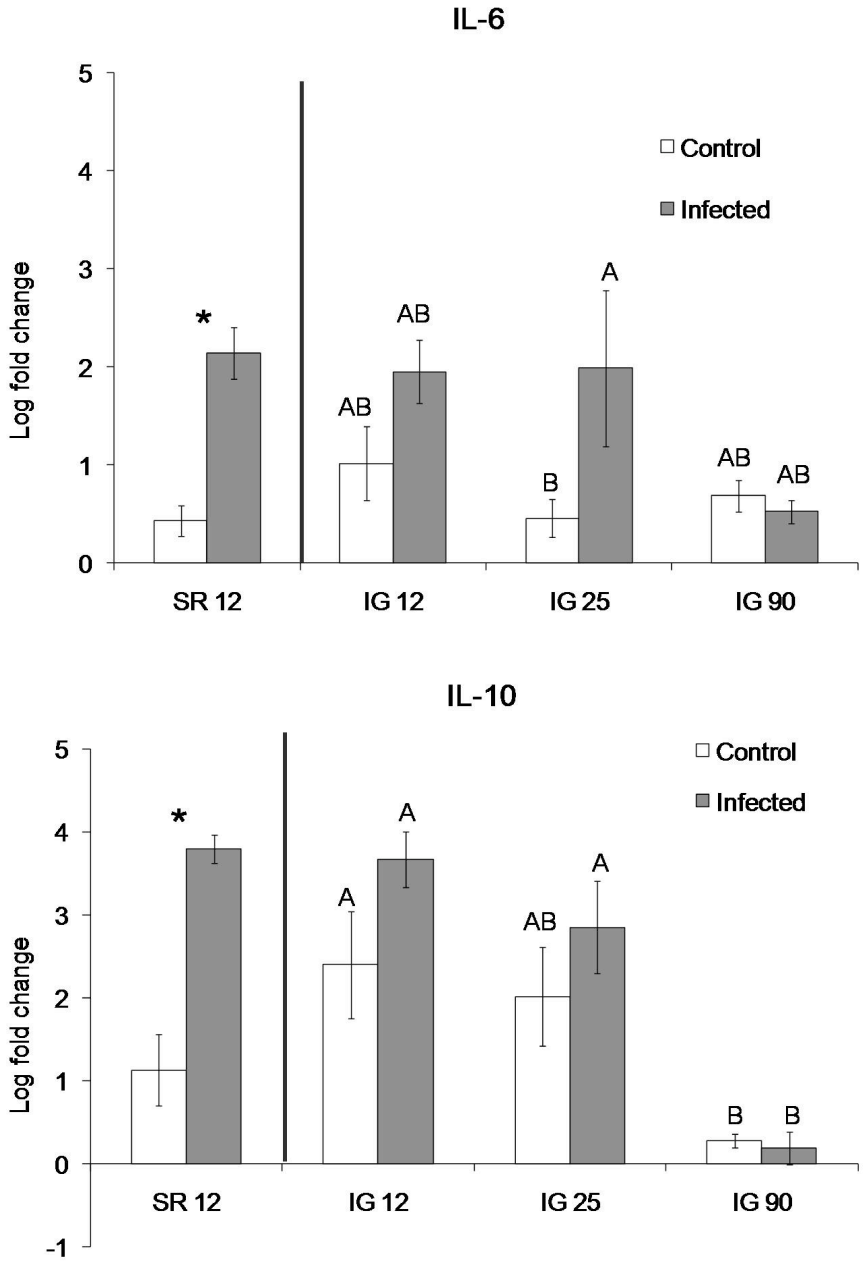
IL-6
Log fold change
5
4
3
2
1
0
Control
Infected
*
AB
AB
A
B
AB
AB
SR 12
IG 12
IG 25
IG 90
IL-10
Log fold change
5
4
3
2
1
0
-1
Control
Infected
*
A
A
A
AB
B
B
SR 12
IG 12
IG 25
IG 90

Figure 7.5

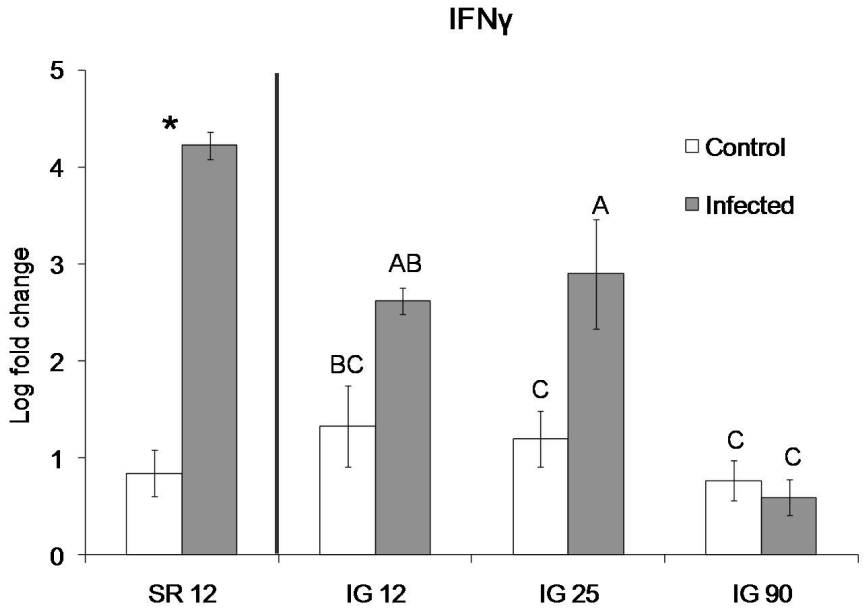

Figure 7.5 (continued). Average log fold change in the expression of IL-6, IL-10 and IFNγ in the intestine of SR and IG Chinook salmon over the course of the infection. SR 12 indicates SR Chinook salmon 12 days p.i. IG 12, IG 25 and IG 90 indicates IG Chinook salmon, 12, 25, or 90 days p.i. respectively. Error bars indicate the standard error. Asterisk indicates significant difference between control and infected SR, letters indicate significant differences between IG infected and control over time.

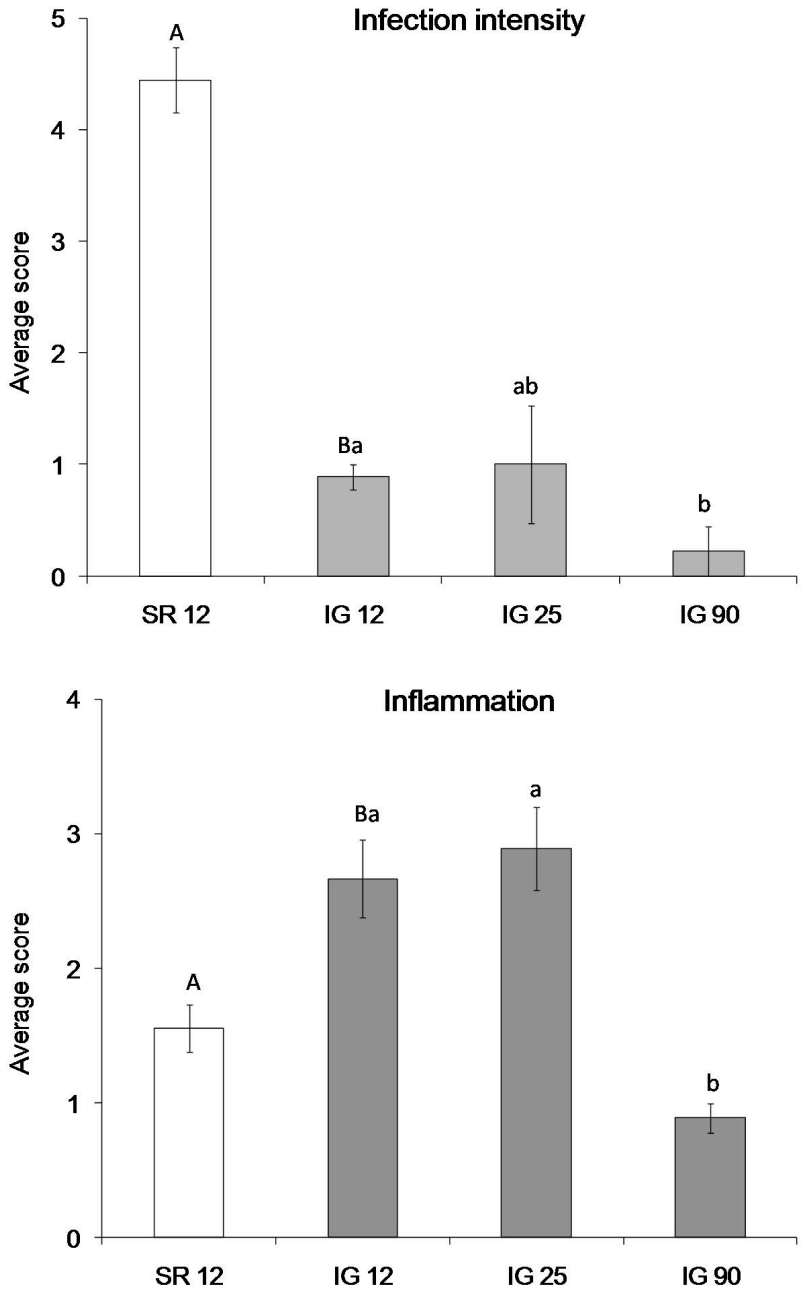

Figure 7.6 Average infection intensity and inflammation scores for Salmon River (SR) and Iron Gate (IG) Chinook salmon infected with *Ceratomyxa shasta*. SR 12 indicates SR Chinook salmon 12 days p.i. (white) IG 12, IG 25 and IG 90 indicates IG Chinook salmon (gray), 12, 25, or 90 days p.i. respectively. Error bars indicate the standard error. Uppercase letters indicate significant difference between SR and IG at day 12, lower case letters indicate significant differences between IG over time.

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ACKNOWLEDGEMENTS

We sincerely thank Adam Ray and Rich Holt for their assistance with fish exposures and fish care as well as Beaver Creek RV Park for land access to our field site. We thank Don Stevens, Harriet Lorz and Charlene Hurst for maintaining polychaetes in the lab as well as with fish care and sampling; and Gerri Buckles for running QPCR. Help from Julie Alexander and Lan Xue on statistical analysis was much appreciated. ODFW is thanked for Salmon River Hatchery fish and California Fish and Game for salmon from Iron Gate Hatchery, CA. This work was funded by Oregon Sea Grant.

CHAPTER 8: SUMMARY

The goal of this study was to identify factors that affected the transmission of *C. shasta* between *M. speciosa* and fish hosts. In the Klamath River, *C. shasta* is associated with high mortality of Chinook and coho salmon and there is great interest in developing management strategies that decrease the incidence and severity of ceratomyxosis in these fish populations. The overlap of hosts is a primary determinant for the continuation of the *C. shasta* life cycle and limiting the size of the polychaete population in the river would have a direct affect on the continuation of the *C. shasta* life cycle. High flushing flows to move sediment and disrupt *M. speciosa* habitat through scouring as well as dropping water levels to expose and dry out this habitat have been proposed as strategies to control the distribution and density of this host. This study determined that *M. speciosa* survives better in water with 0.03 m/s velocity in comparison to 0.01 m/s, making it more likely to find more abundant and denser populations at the higher velocity than the slower. Temperature did not affect the survival of *M. speciosa* infected with *C. shasta*, and we expect that this holds true for non-infected *M. speciosa* as well. Additionally, polychaetes can survive a 24 h de-watering event (Appendix 1), although the survival was very low. Thus, a brief drop in water level is likely to eliminate only some of the polychaetes, and warm water temperatures that could accompany the shallow water generated in such an event are unlikely to affect the survival of this host at all. Knowledge of how *M. speciosa* populations respond to fluctuations in velocity, the stability of their habitat at varying velocity and sediment loads in the water column, as well as the rate in which they colonize an area would assist in identifying velocities that would decrease polychaete population density and abundance and determine where new populations would arise.

The transmission of myxospores to *M. speciosa* and the development and release of actinospores is the next step in continuation of the *C. shasta* life cycle. Water velocity affected the delivery of myxospores to *M. speciosa* resulting in higher infection prevalence at slower water velocities. Once infection in *M. speciosa* has initiated, the maturation and release of the actinospores is temperature dependent.

Actinospores develop in the dermis of *M. speciosa* and are released through a mucus pore into the water. *Ceratomyxa shasta* can over winter in *M. speciosa* and increasing temperature initiates actinospore maturation and release. When applied to a seasonal infection model, spawning adults shed myxospores in the fall and winter infecting polychaetes that harbor the infection in an arrested state until temperatures warm in the spring and actinospores are released infecting juveniles. Juveniles also release myxospores, and at warm temperatures *C. shasta* actinospores can develop in and be released from *M. speciosa* in 2 months, which would align with the return of adults to freshwater in late summer and into the fall. Infected *M. speciosa* can maintain the infection for at least 30 days (at 4-20ºC), thus, these worms may remain infected throughout the spring and summer and continue to release actinospores as well.

It is unknown if the infection intensity of *C. shasta* in *M. speciosa* is affected by temperature. If infection intensities are higher at warmer temperatures, then warmer water temperatures would result in even higher actinospore doses and would occur even earlier. Infective dose was not affected by fish size (in susceptible strains of rainbow trout), so earlier release should not affect the susceptibility of the fish host, however, higher actinospore concentration definitely would affect the infection prevalence and infection severity in fish.

Once actinospores are released into the water, they must remain viable long enough to encounter a fish host. Actinospore viability decreases with increasing temperature over time, but actinospores can remain viable for up to 18 days at 4ºC and 6 days at 20ºC. Thus, at 20ºC, actinospores would be released in great numbers, but survive for a shorter period of time. Subsequent infection of fish would depend on what that concentration is and the infectious dose for that strain of fish. In the river, this means that at warmer temperatures, the parasite concentration in an infectious zone is expected to be higher than at cooler temperatures, but the infectious area down stream of the infected polychaete population would be smaller. At cooler temperatures, fewer actinospores would be released but would survive longer and be distributed further down stream. The affects of either scenario on fish infection depends on the initial concentration of the actinospores and the influence of tributaries and springs that result in parasite dilution. In this study, the affect of water

velocity on the delivery of actinospores to fish was confounded by parasite dose and the effect of this parameter on transmission to fish should be investigated in the future.

Actinospores must be present in great enough numbers to overcome the fish's defenses in order to develop into myxospores. The parasite attaches to the gill epithelium, migrates between epithelial cells, replicates in a blood vessel and migrates the intestine where it proliferates further. There were no differences in the invasion of parasite into the gills of either susceptible or resistant strains of fish. Parasite genotype is a confounding factor in the interpretation of dose and resulting disease. One actinospore (from polychaetes infected with actinospores from rainbow trout, presumably genotype II) is sufficient to induce a lethal infection in susceptible rainbow trout but resistant Chinook and coho salmon resisted ceratomyxosis when exposed to as many as 5,000 of these actinospores. Resistant Chinook salmon became infected with and recovered from *C. shasta* when infected with sub-lethal doses in the range of 4,300 (Chinook genotype I) to 165,000 (mix of genotypes I and II) actinospores per fish. In these fish, *C. shasta* was eliminated from the blood, developmental stages were arrested in inflammation in the intestine then subsequently eliminated and trophozoites were expelled into the lumen of the intestine prior to maturation into myxospores. It is unknown if trophozoites continue to mature outside of the fish tissue, so the survival of these parasite stages is unknown.

Although the fate of the different *C. shasta* genotypes in non-specific hosts has not been determined in natural infections, it is possible that the resistant Chinook and coho salmon were infected by the rainbow trout genotype II actinospores, but resolved the infection. A susceptible strain of Chinook salmon suffered fatal infections when injected with type II and III trophozoites from rainbow trout whereas no resistant salmon succumbed to the infection or showed clinical signs (Appendix 2). Thus, non-host specific genotypes can cause disease in susceptible hosts, but these same genotypes may infect but be eliminated from resistant hosts making non-susceptible hosts a "sink" for these actinospores.

In addition to understanding the transmission of this parasite between hosts, this study identified protective mechanisms resistant fish used to survive *C. shasta*

infection. This study found evidence of the Th2 (adaptive immune response) as well as parasite tolerance in resistant Chinook salmon that recovered from *C. shasta* infection. An antibody response to *C. shasta* has been discovered in susceptible rainbow trout and is suspected to play a role in the response of resistant fish as well. Some fish develop antibodies against a parasite that results in the expulsion of the parasite and this response is protective against future infection. This parallels the observations of immature parasites in the lumen of the intestine of fish surviving *C. shasta* infection. It is not known if an antibody response is involved in these fish or if it is protective but studies should focus on this area in the future. If the response is protective, the dose necessary to elicit this response should be identified as well as the threshold of parasite doses for which this response remains protective. Knowledge of these factors could be used to create flow regimes that facilitate parasite doses that induce a protective response for fish. Information on the threshold for the protection of the response would also enhance the accuracy of predictive models.

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Appendices

APPENDIX 1: THE EFFECTS OF TEMPERATURE AND DEWATERING ON THE SURVIVAL OF *MANYUNKIA SPECIOSA*

ABSTRACT

This study investigated the effects of water temperature and de-watering on the survival of the polychaete in its two primary substrates, *Cladophora* sp. and a mixture of sand and silt in a flow through experimental design. An inverse relationship between temperature and polychaete survival was observed in these substrates using a flow through well water culture method. However, overall survival was low likely due an insufficient food source. A small percentage of polychaetes survived 24 h de-watering in both substrates as measured at 12 weeks, but at least 43% of polychaetes in sand silt can survived for 4 weeks in the sand silt substrate. It is surprising that any polychaetes survived the drying event, with improved culture conditions we suspect that survival would be even higher. These results indicate that as water height fluctuates in the river both seasonally and by man-made draw downs polychaete populations may decline slightly but may leave enough survivors to re-colonize the area.

Keywords: *Ceratomyxa shasta*, polychaete, *Manayunkia speciosa*, de-water, temperature, survival

INTRODUCTION

Manayunkia speciosa, is the definitive host of *Ceratomyxa shasta* (Bartholomew et al., 1997) a myxosporean parasite of salmonids. It is a small freshwater tube dwelling sabellid polychaete worm with broad distribution in the benthos of freshwater lakes, streams, and rivers across North America. Mackie and Qadri (1971) summarize the distribution of *M. speciosa* along the east coast from New Jersey down to North Carolina, across the northern Midwest in Wisconsin and Ontario, Canada and in the west from as far north as Alaskan lakes and south to the Sacramento River, CA. *Manayunkia speciosa* inhabits a versatile range of temperatures (from 4 to > 25°C) and flow regimes (<0.01 to 3.0 m/s) (Mackie and Qadri, 1971; Holmquist, 1973; Stocking and Bartholomew, 2007). This host is generally described as colonizing soft sediments such as sand with a mix of fine benthic matter or on algae covered rocks that collect this material (Pettibone, 1953; Hazel, 1966; Mackie and Qadri, 1971). *Manayunkia speciosa* reproduces sexually or asexually, creating broods of up to 40 offspring (Willson et al., in press), but the rate of reproduction and lifespan of these worms are unknown (Croskery, 1978; Stocking and Bartholomew, 2007). Increased knowledge about the survival of this host in response to changing environmental parameters will enhance our understanding of the population dynamics of this host which will in turn provide information on the establishment and persistence of *C. shasta* in an area.

The presence and density of a *M. speciosa* population depends upon the suitability of the available habitat and food abundance. Stocking and Bartholomew (2007) report stable substrate, moderate flows, and fine benthic organic matter as the defining characteristics of *M. speciosa* habitat in the Klamath River Basin. They hypothesize that the absence of *M. speciosa* populations in areas of available habitat is due to poor dispersal and insufficient food base. Seasonal changes in precipitation affecting flow rate and water temperature and may vary greatly between summer and winter months. Irrigation and hydroelectric demands also influence water level and flow patterns for many rivers. The effects of water flow on *M. speciosa* survival and *C. shasta* infection were studied in Bjork and Bartholomew (2009). The effects of drying of substrate on a *M. speciosa* population are unknown, and thus far only the

effects of temperature on a population of *M. speciosa* with high *C. shasta* prevalence have been tested (Chapter 2).

This study examined the effect of 3 different temperature regimes on the survival of *M. speciosa* populations with natural infection prevalence in two of its primary habitats, *Cladophora* spp. and a mixture of sand and silt. Additionally, the effect of a 24h de-watering event on the survival of the same initial population of *M. speciosa* was tested. A flow through well water culture method was used for maintenance of the polychaetes for the duration o the experiment.

MATERIALS AND METHODS

Collection and maintenance of polychaetes: Polychaetes were collected from the Klamath River system from two substrates in which they are commonly found. The epiphytic algae *Cladophora* was collected at river kilometer (RKm) 292 (I-5 site) (Fall 2005). The sand-silt substrates were collected from the mouth of the Williamson River (RKm 0), a tributary of Klamath Lake (Spring 2006). These collection sites are geographically distant, and due to this separation, these populations may be genetically distinct. However, environmental variables such as temperature and precipitation in each of the locations are similar; therefore we expect the physiological responses of these populations to be comparable. To date, there are no reports of differences in the tolerance of *M. speciosa* to changing environmental parameters from different locations. Samples were collected using an 83 µm mesh plankton net (17 cm diameter) fitted on a telescoping handle. A 2 cm metal flange was attached to the rim of the net to facilitate scraping of hard substrates. A plastic kitchen spatula was also used to scrape *Cladophora* off of rocks. Material was placed in plastic bags with approximately 500 ml of Klamath River water, supplied with oxygen via an airstone and transported back to the John L. Fryer Salmon Disease Laboratory (SDL), Oregon State University, Corvallis, OR. All experiments were carried out at the SDL using specific pathogen free (SPF) well water unless indicated otherwise.

Division of substrate into replicates: Individual bags of each substrate (*Cladophora* sp. and sand-silt) were pooled according to substrate type and mixed for randomization. Polychaete density within *Cladophora* is highly variable, so this

substrate was homogenized by breaking the algae into 2 cm squares and mixing before distribution into 500 ml containers. Equal amounts of each substrate type and associated biota (30 ml aliquots of *Cladophora* containing an average of 147 ±18 (standard deviation, SD) polychaetes per container and 50 ml of sand-silt with an average of 197 ±52 (SD) polychaetes per container) were distributed in shallow plastic containers in triplicate. Two additional replicates for each treatment (5, 13, or 20°C or 24 h de-watering) were created for determination of polychaete density at 4 and 8 weeks (only one replicate for each of these time points). Each container was fitted with a hole to facilitate a low flow (approximately 100 ml/min) of water. Experiments I and II were run in tandem.

Quantification of polychaetes: Polychaetes were quantified at 65x magnification using a dissecting microscope. The entire 30 or 50 ml samples collected from the temperature, dewatering, and flow experiments were examined. The preserved substrate was emptied into a Petri dish in 10 ml increments and modified dental tools were used to separate polychaetes from the substrate. Higher magnification was used as necessary to confirm polychaete identification. Due to the possibility of polychaete replication and inability to monitor the survival of individual polychaetes, the relative change in polychaete survival (RPS) was used to measure changes in population densities throughout the experiment. The RPS was calculated for each sample by comparing the density of polychaetes in each treatment group and time point with the mean density of the initial sample of the respective substrate.

Experiment I. Temperature effects on polychaete survival in a flow through well water system: To test the effect of temperature on *M. speciosa* survival, water at 5, 13, or 20°C was supplied 5 of the containers of each substrate for the duration of the 3 month experiment or until termination. One replicate was fixed in ethanol at weeks 4 and 8 for polychaete density determination. At 12 weeks, all material was fixed in ethanol for polychaete density determination.

Experiment II. The effects of a 24h drying event of the habitat on polychaete survival: Polychaetes in sand-silt and *Cladophora* substrates were collected, transported and divided into replicates in the same volumes as in Experiment I. To simulate a de-watering or drying event, water was poured off of the containers containing substrate and

polychaetes and it was allowed to dry under a grow light (Sylvania Gro-Lux Standard 1.5 in diameter, 40 Watts) for 24 h with the temperature reaching 25° C. After 24 h, a low flow (approximately 100 ml/min) of water at 13°C was supplied to each of the containers for the duration of the 3 month experiment or until termination. Density determination was performed as in Experiment I.

RESULTS

Experiment I. Temperature effects on polychaete survival in a flow through well water system: Polychaete densities in the sand-silt substrate at 5 and 13˚C remained relatively stable during the first four weeks of the experiment, with a relative percent survival (RPS) of 66 and 93%, respectively (Figure 1). Densities of polychaetes held at 5˚C remained stable over the experimental period compared to the 13 and 20˚C treatment groups, with a RPS of the three replicate groups at 12 wks of 32, 62, and 69 %. Survival at 13˚C decreased more markedly, with RPS of 14, 23, and 29% at 12 wks. Polychaetes held at 20˚C had high mortality during the initial 4 wks (27% RPS) and at 12 wks survival was 2% or less for the 3 replicates.

There was a large decline in polychaetes in *Cladophora* between the initiation of the experiment and the first sampling at wk 4 at all three temperatures (Figure 1). At this time, RPS was 22% for those held at 5˚C while RPS at 13 and 20˚C was less than 5%. By wk 12 no significant recovery was observed at any temperature in this substrate.

Experiment II. The effects of a 24h drying event of the habitat on polychaete survival: Following a 24 h period of dewatering and high temperature, there was a significant decline in the number of surviving polychaetes in both substrates (Figure 2). At 4 wks, the RPS of *M. speciosa* in *Cladophora* was only 2%, compared with 42% for the sand-silt treatment. Polychaetes were not detected from subsequent samples of the *Cladophora*. Although survival of polychaetes in sand-silt continued to decline, 25% remained at 8 wks and RPS at 12 wks was 3, 13 and 19%.

DISCUSSION

It has been noted that polychaetes do not inhabit edge habitats where water levels fluctuate (Stocking and Bartholomew, 2007), and it could be expected that an organism with the apparent fragility of the polychaete would be unable to survive even a brief period of drying. Although dewatering for 24h significantly reduced the number of

polychaetes that survived in the sand- silt habitat, nearly 43% survived the event. Changes in survival after that time may have been influenced by water temperature as well, as 13°C water was supplied at low flow for the remainder of the experiment. From the temperature experiment, polychaetes in sand-silt held at 13°C, experienced a 10% decline in density in the first 4 weeks. If temperature caused the same effect in the dewatering experiment, only 50% of the total loss in sand-silt can be attributed to dewatering and the other 10% may be a temperature effect. Previous observations indicate that there are no resting stages (Holmquist, 1973), so survival under these drying conditions is remarkable. Although the substrate in this experiment was not completely dry and some moisture remained, the polychaetes did survive static conditions at temperatures as high as 25 °C. Because the rate of maturity and lifespan of *M. speciosa* is not known, it is unclear whether one life stage is more vulnerable to these conditions than others.

While the effects of temperature and dewatering on polychaetes in the sand-silt habitat were clear, we were unable to draw conclusions on how these parameters might effect populations in *Cladophora.* The immediate decline in polychaete densities in the *Cladophora* habitat used in both experiments is likely to have been affected by increased substrate disturbance. The sand silt mixture settles more quickly than *Cladophora,* possibly generating a more stable environment. The removal of *Cladophora* from rocks and boulders in the river, followed by the division of this mat-like algae to reduce variations in polychaete density, likely damaged polychaete tubes and marginalized its habitat. Because few polychaetes survived under any conditions in *Cladophora,* confounding variables such as these may have overshadowed the effects of temperature and dewatering in this study.

In both of the temperature and dewatering experiments, the absence of an adequate food source may have also contributed to the decline of populations in both substrates. Algae and bacteria that grew in the sample container were the only food sources available to the polychaetes in this experiment. If food was indeed the factor limiting survival, the increased survival at 5°C could be due to a reduction in metabolic rate at this temperature. Considering the survival of *M. speciosa* in shallow Alaskan

lakes where water reaches or remains just above freezing in the winter (Holmquist, 1973), such a strategy seems plausible for over-wintering.

The findings of this study indicate that the changing parameters of the environment influence the survival of *M. speciosa.* Temperature had an inverse relationship with polychaete survival but survival was low in all treatment groups indicating that this culture method was unsuccessful most likely due to an inadequate food source. Additionally, a 24 h drying event greatly decreases polychaete survival, but does not eradicate them. It is surprising that any polychaetes survived the drying event; with improved culture conditions we suspect that survival would be even higher. These results indicate that as water height fluctuates in the river both seasonally and by man-made draw downs polychaete populations may decline slightly but may leave enough survivors to re-colonize the area.

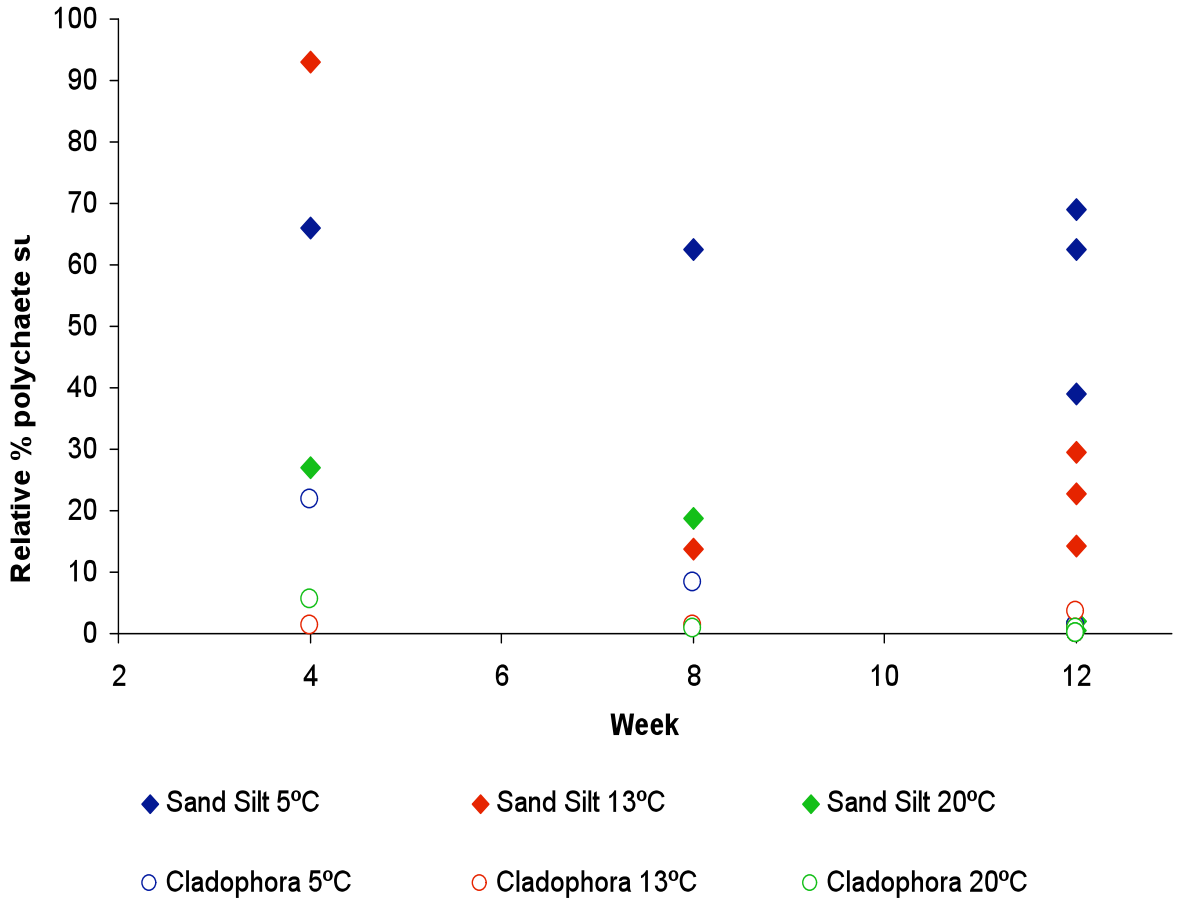

Figure 1. *Manayunkia speciosa* survival in two substrates: *Cladophora* and a mixture of sand and silt at 5, 13, and 20ºC in a flow through well water culture system.

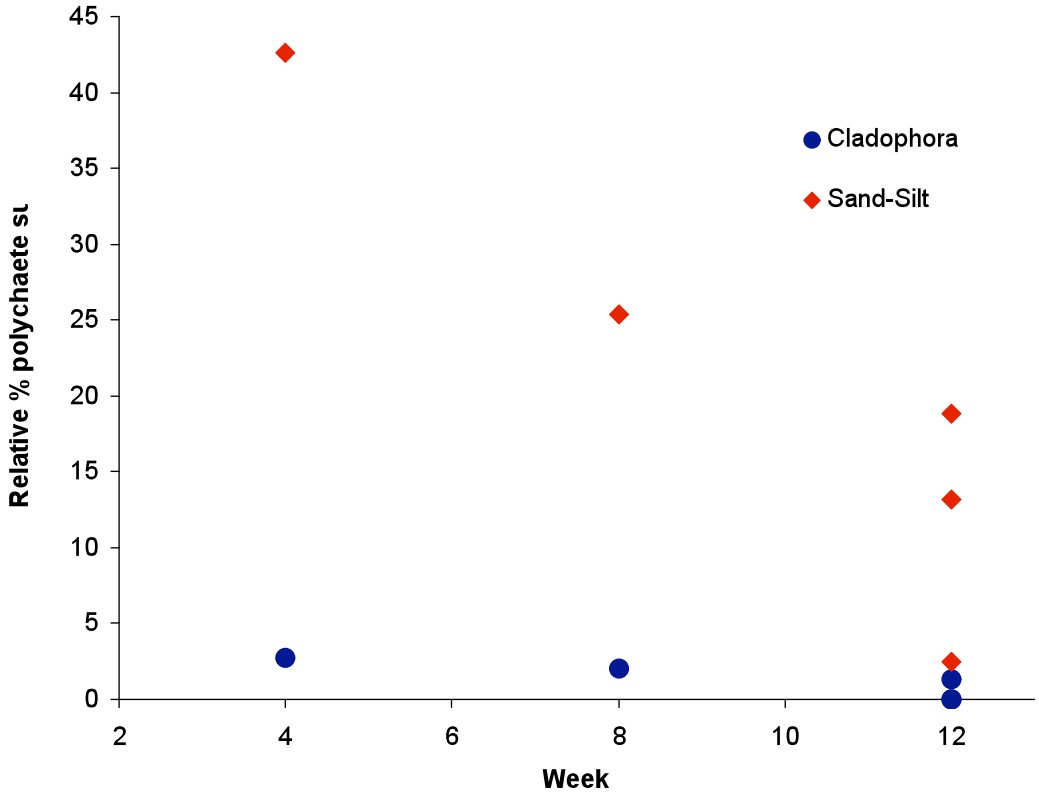

Figure 2. *Manayunkia speciosa* survival in two substrates: *Cladophora* and a mixture of sand and silt after a 24h de-watering event.

APPENDIX 2: INFECTION OF SUSCEPTIBLE AND RESISTANT STRAINS OF CHINOOK SALMON WITH NON-HOST SPECIFIC *CERAOMYXA SHASTA* GENOTYPES

ABSTRACT

Recent studies have identified genetic differences in *Ceratomyxa shasta* isolated from different salmonid host species and geographic regions within the same river basin. It is not known if the full spectrum of genotypes is absent in certain fish species because the parasite simply does not infect that species, or because the fish are capable of eliminating the infection of some genotypes. In this study, we investigate the response of Chinook salmon (*Oncorhynchus tshawytscha*), to two *C. shasta* genotypes (Types II and III) that have only been associated with pathogenicity in rainbow trout (*Oncorhynchus mykiss*). To determine if the fish host response to the infection is the driving factor behind the trends reported, two strains of Chinook salmon, a highly susceptible (S) resistant strain (R) were injected with trophozoites from an infected rainbow trout and the infection severity was compared. Rainbow trout were included as a control and all succumbed to infection; a higher proportion of S Chinook succumbed to infection than R Chinook, even though the infection prevalence was similar between the 2 strains. Although the natural route of infection was bypassed, this study demonstrates that Chinook salmon can succumb to infection with *C. shasta* genotypes II and III. It also demonstrates that in resistant Chinook salmon these genotypes are not pathogenic.

Keywords: Chinook salmon, rainbow trout, resistant, *Ceratomyxa shasta*, genotype, injection

INTRODUCTION

Recent studies have identified genetic differences in *Ceratomyxa shasta* isolated from different salmonid host species and geographic regions within the same river basin. Atkinson and Bartholomew (in press) have identified 4 distinct genetic sequences in the ITS region of the *C. shasta* genome. The Type 0 is reported only in steelhead, Type I and II in Chinook salmon, and Types II and III in coho and rainbow trout. Interestingly, disease in Chinook salmon coincides only with the presence of Type I. Chinook salmon infected with type II show no clinical signs, whereas rainbow trout infected with Type II experience high rates of mortality. The discrepancy between infection severity among different fish species infected with the same genotype implies possible host-species specific pathogenicity of the parasite. The inherent resistance of the fish host complicates drawing this conclusion and interpretation of the findings of Atkinson and Bartholomew (in press).

Atkinson and Bartholomew's study was confined by the strains of fish available; resistant Chinook salmon, resistant coho salmon and both susceptible and resistant rainbow trout. Increased resistance of fish from areas where the parasite is endemic is well established (Bartholomew 1998), because no susceptible stains of fish were tested besides rainbow trout, it is unknown if the genotypes infecting these fish are truly fish host-species specific. To determine whether fish host species or fish host resistance is the driving factor behind the trends reported, the infection of two strains of Chinook salmon, a highly susceptible (S) and a resistant strain (R) are compared when challenged with *C. shasta* from rainbow trout.

MATERIALS AND METHODS

Fish Twenty Chinook salmon were obtained from the Salmon River Hatchery, OR as the susceptible (S) strain. Twenty Chinook salmon were also obtained from Iron Gate Hatchery, CA as the resistant (R) strain, and 20 Roaring River rainbow trout (highly susceptible strain used as a positive control) were transported to the John L. Fryer Salmon Disease Laboratory at OSU. Fish were maintained in 100 L flow through tanks supplied with specific pathogen free well water and fed a commercial fish food diet daily.

Parasite source: Trophozoites were obtained by aspirating the ascites of a fatally infected rainbow trout that had become infected after challenge to *C. shasta* through

exposure to infectious Willamette River (Corvallis, OR) water. Trophozoites were purified by three washes in PBS (centrifuged at 1000 rpm for 5 minutes each) and re-suspended in 7.5 ml. Trophozoite density was estimated by averaging three counts of the parasites using a hemacytometer. Fish were anesthetized with Na CO_3 buffered MS222 (5 g/L) and injected in the peritoneal cavity with 4.38 x 10^4 parasites in 0.1 ml. Five control fish of each stock were injected with the same volume of PBS that did not contain trophozoites. Ten fish of each injected group were reared at 18°C and the other ten were reared at 13°, except for the control fish which were reared at 18°C. A portion of the ascites was reserved for parasite DNA amplification and sequencing (Atkinson and Bartholomew in press). Fish were monitored daily, and infection prevalence in mortalities was confirmed by a wetmount of an intestinal scraping and visualization of myxospores. When myxospores were not observed, a piece of posterior intestine was removed and frozen until analysis by PCR (Palenzuela et al., 1999). After 60 days, all remaining fish were euthanized with an overdose of MS222, samples of intestine were collected for PCR and fixed in Davidson's fixative for histology. Histology slides were prepared by routine methods by the Veterinary Diagnostic Lab at OSU and stained with Giemsa.

RESULTS

All of the rainbow trout injected with trophozoites died prior to the termination of the experiment, and only one fish from the 13°C group, and two fish from the 18°C group were not producing myxospores. These fish were assayed by PCR. 60 % of the 18°C S Chinook had fatal infections, 10% of the S at 13°C and none of the R Chinook died at any temperature (Figure 1). Of the fatally infected fish, fish at 18°C died earlier than 13°C fish (Figure 1). PCR analysis detected *C. shasta* DNA in 70% of R at 13°C and 50% of R at 18°C. Seven of the 8 SR at 13°C assayed positive by PCR, making the total prevalence 88% and one of the surviving SR 18°C S assayed positive making the total prevalence 70%. PCR analysis of the rainbow trout resulted in total prevalence of 90% for both the 13 and 18°C groups. Histology of PCR positive fish revealed no observable inflammation or parasites in the intestine of surviving S or R Chinook.

DISCUSSION

This study provided no evidence toward fish host specificity for *C. shasta* genotypes. Both strains of Chinook salmon were infected with rainbow trout Type II and III *C. shasta* and S Chinook suffered fatal infections. Thus, for Type II and III at least, fish host resistance appears to play a bigger role than parasite genotype. Although the i.p. route of infection in this study was artificial, the fact that some S Chinook succumbed to infection demonstrates that Types II and III are pathogenic in the *Oncorhynchus tshawytscha* species. The lack of any mortality but persisting infection prevalence in R Chinook indicates that the resistance of this stock is capable of containing the Type II and type III genotypes.

R Chinook do succumb to infection with Type I, so this genotype must have some virulence traits distinct from types II and III. It is unknown if other species such as rainbow trout and coho salmon can become infected with type I, and testing this in field studies is difficult due to the presence of genotype II at all locations where genotype I is also found. The detection of Types II and III in the intestine of susceptible rainbow trout, even when type I is in the water would indicate that II and III proliferate faster than type I. If types II and III are truly faster growing, it further demonstrates the ability of the R Chinook salmon to eliminate infection with these more aggressive genotypes. However, type I should not be considered less virulent, as it causes mortality in even resistant Chinook salmon at high enough doses. Thus, Type I may be slower growing and less competitive than types II and III.

The pathogenic mechanisms used by these genotypes are completely unexplored. Ceratomyxosis is manifested similarly between fish host species; inflammation and hemorrhage in the gut, necrosis and fish host death. Therefore, the pathogenic effects of the genotypes are not likely to be that different, but the timing and mechanics may be different. Further investigation of the physiology of the different genotypes is likely to reveal differences in surface antigens that lead evasion of the fish host immune system or differences in timing or potency of protease production.

In addition to ruling out species specific pathogenicity of rainbow trout genotypes, this study reinforced previous findings on host response to *C. shasta* infection. Previous studies with intraperitoneal injections of parasites in fish have shown that inflammation and isolation of the parasite in granulomata have been effective in

containing the parasite (Ibarra et al., 1991a; Foott et al., 2004b). The advance of type II and III trophozoites in the intestine of S Chinook salmon demonstrates that progress of infection and myxospore formation can progress demonstrating that Chinook salmon are not aberrant hosts for these genotypes. It is more likely that the parasites invade any fish host available and their fate is determined by the response of the fish host. Since R Chinook can ultimately succumb to infection with genotype I but not type II, there are differences in the pathogenicity of these genotypes which are yet unknown.

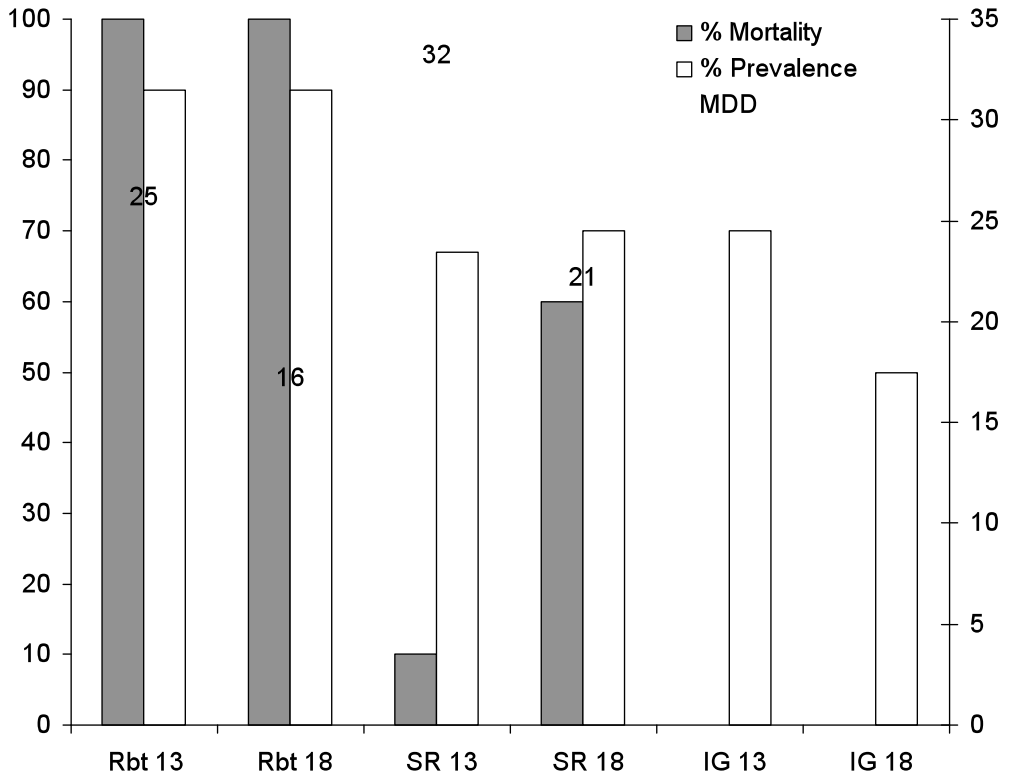

Figure 1. Infection prevalence, mortality and mean day to death of susceptible rainbow trout (Rbt), a susceptible strain of Chinook salmon (SR) and a resistant strain of Chinook salmon (IG) at 13 and 18 ºC.