Ceratomyxa shasta-related Concerns for Reintroduced Anadromous Salmonids into the Upper Klamath Basin, CA-OR, USA
AN ABSTRACT OF THE THESIS OF

Charlene N. Hurst for the degree of Master of Science in Microbiology presented on December 7, 2010.
Title: Ceratomyxa shasta-related Concerns for Reintroduced Anadromous Salmonids into the Upper Klamath Basin, CA-OR, USA

Abstract approved: ________________________________

Jerri L. Bartholomew

The myxozoan parasite of salmonids, Ceratomyxa shasta, is established throughout the Klamath River, CA-OR, with high parasite densities below the series of dams and above the dams (upper basin) in the northernmost tributary, the Williamson River (WMR). Two objectives were designed to address concerns about C. shasta effects on reintroduced anadromous salmonids in the upper basin. The first objective investigated parasite dynamics in the WMR and the second determined the virulence of parasite genotypes in allopatric and sympatric salmonid strains. Parasite density, distribution and genotype composition were assessed from water samples collected throughout the WMR and its tributaries. High parasite densities occurred in two sections of the WMR and the genotype associated with stocking of an allopatric strain of rainbow trout predominated. In laboratory cross-infection experiments, the stage of the parasite infective for fish of genotypes I and II (biotype IIR and IIC) were used in three separate trials to challenge four strains of salmonid; sympatric strains of Chinook (Oncorhynchus tswytschcha) and coho (O. kisutch) salmon and allopatric strains of Chinook salmon and rainbow trout (O. mykiss). Genotype I caused mortality in both Chinook strains, while mortality in the allopatric Chinook strain also occurred with IIR. Mortality of rainbow trout resulted from biotypes IIR and IIC, but mortality in coho only occurred with exposure to IIC. Thus, the parasite genotypes/biotypes that cause mortality in native Klamath River fish (i.e. I and IIC) were not present in the upper basin, but could become established and amplify as the stocking of allopatric rainbow trout illustrates.
Ceratomyxa shasta-related Concerns for Reintroduced Anadromous Salmonids into the Upper Klamath Basin, CA-OR, USA

by

Charlene N. Hurst

A THESIS

Submitted to

Oregon State University

In partial fulfillment of the requirements for the degree of

Master of Science

Presented December 7, 2010
Commencement June 2011
Master of Science thesis of Charlene N. Hurst presented on December 7, 2010

APPROVED:

Major Professor, representing Microbiology

Chair of the Department of Microbiology

Dean of the Graduate School

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

Charlene N. Hurst, Author
ACKNOWLEDGEMENTS

The author expresses sincere appreciation for all of my family, friends and coworkers for their support over the past two years. Special thanks to my parents for their encouragement through all of my schooling and to my boyfriend Shawn Harris, for “volunteering” to help with various field projects and lab work. I would like to thank all the members of the Bartholomew lab for listening to various ideas, reviewing experiments, helping with data analysis and for making my experience here at Oregon State University enjoyable and unforgettable. Thank you to all my committee members for reading this lengthy document and for their constructive criticism; especially my advisor Jerri Bartholomew, for reviewing the many drafts of both manuscripts before their inclusion in this thesis.
CONTRIBUTION OF AUTHORS

Dr. Bartholomew served as major advisor and contributed to the experimental design and writing of manuscripts. Dr. Holt assisted with data collection and analysis in Chapter 2.
# TABLE OF CONTENTS

1 Introduction .................................................................................. 1  
   The Parasite ................................................................................. 2  
   The Hosts .................................................................................... 3  
   *Ceratomyxa shasta* in the Klamath River System ......................... 6  
   Goals and Objectives ................................................................ 9  

2 *Ceratomyxa shasta* Dynamics in the Williamson River:  
   Implications for Reintroduced Anadromous Salmonids ............... 13  
   Abstract ...................................................................................... 14  
   Introduction ................................................................................ 15  
   Materials and Methods .............................................................. 17  
   Results ......................................................................................... 21  
   Discussion .................................................................................. 23  
   Acknowledgements .................................................................... 27  
   References .................................................................................. 28  

3 Virulence of *Ceratomyxa shasta* Genotypes I and II in Allopatric  
   and Sympatric Fish Strains ......................................................... 36  
   Abstract ...................................................................................... 37  
   Introduction ................................................................................ 38  
   Materials and Methods .............................................................. 40  
   Results ......................................................................................... 43  
   Discussion .................................................................................. 45  
   Acknowledgements .................................................................... 50  
   References .................................................................................. 51
# TABLE OF CONTENTS (Continued)

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 Summary</td>
<td>62</td>
</tr>
<tr>
<td>Summary</td>
<td>63</td>
</tr>
<tr>
<td>Management Considerations</td>
<td>64</td>
</tr>
<tr>
<td>Future Research</td>
<td>65</td>
</tr>
<tr>
<td>Bibliography</td>
<td>67</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td><em>Ceratomyxa shasta</em> life cycle</td>
<td>11</td>
</tr>
<tr>
<td>1.2</td>
<td>The Klamath River Basin with the Williamson River outlined and enlarged to the right</td>
<td>12</td>
</tr>
<tr>
<td>2.1</td>
<td>Williamson River and tributaries with the Klamath River basin in the left insert</td>
<td>31</td>
</tr>
<tr>
<td>2.2</td>
<td><em>Ceratomyxa shasta</em> cycle threshold (Cq) values and temperatures for Williamson River water samples collected in 2008, 2009 and 2010 by reach</td>
<td>32</td>
</tr>
<tr>
<td>2.3</td>
<td>Quadratic regression plot of temperature versus <em>Ceratomyxa shasta</em> cycle threshold (Cq) values for all months and reaches</td>
<td>33</td>
</tr>
<tr>
<td>2.4</td>
<td><em>Ceratomyxa shasta</em> parasite genotype composition from water samples collected in the Williamson River during all four sampling periods</td>
<td>34</td>
</tr>
<tr>
<td>2.5</td>
<td>Mortality of non-native rainbow trout (bars) and average <em>Ceratomyxa shasta</em> cycle threshold (Cq) values (triangles) for Keno Eddy (sKED) and the Williamson River (sWMR) sentinel sites for years 2006-2010</td>
<td>35</td>
</tr>
<tr>
<td>3.1</td>
<td>Map of the Klamath River basin</td>
<td>54</td>
</tr>
<tr>
<td>3.2</td>
<td>Cross-infection study experimental set-up</td>
<td>55</td>
</tr>
<tr>
<td>3.3</td>
<td>Laboratory cross-infection challenges with genotypes/biotypes of <em>Ceratomyxa shasta</em></td>
<td>57</td>
</tr>
<tr>
<td>3.4</td>
<td>Mean degree day to morbidity of Salmon River Chinook exposed in trial 2</td>
<td>59</td>
</tr>
<tr>
<td>3.5</td>
<td>Mean degree day to morbidity of rainbow trout exposed in trial 3</td>
<td>60</td>
</tr>
</tbody>
</table>
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.1 Fish cross-infection challenge experimental design…………………</td>
<td>56</td>
</tr>
<tr>
<td>3.2 Ceratomyxa shasta parasite genotype identity and genotype proportion in water collected from the polychaete control and treatment aquaria during all three trials…………………………</td>
<td>61</td>
</tr>
</tbody>
</table>
CHAPTER 1: INTRODUCTION
THE PARASITE

Myxozoans are aquatic, eukaryotic, parasites that have a complex life cycle requiring two hosts. The invertebrate host identified so far is an annelid or bryozoan (Kent et al., 2001). Known vertebrate hosts include fish, which comprise the majority, amphibians, ducks and even some mammals such as the shrew and mole (Lom and Dyková, 1996; Friedrich et al., 2000 in Kent et al., 2001; Duncan et al., 2004; Prunescu et al., 2007; Bartholomew et al., 2008). Myxozoan development within the host has only been described for one myxozoan species, Myxobolus cerebralis. In this model the invertebrate host is the site of sexual reproduction and asexual reproduction occurs in the vertebrate host. Transmission of the parasite typically occurs indirectly via the spore stage released from the invertebrate host, but direct transmission from fish to fish has been suggested for Myxidium leei (Diamant, 1997) and Kudoa ovivora (Swearer and Roberts on, 1999).

The myxozoan parasite Ceratomyxa shasta was first characterized by Noble (1950) and is one of only a few known freshwater species of the Ceratomyxa genus (Lom and Dykova, 2006). This parasite requires both a salmonid and polychaete (Manayunkia speciosa) host, in which parasite proliferation and development results in two waterborne spore stages: the myxospore released from the salmonid and the actinospore released from the polychaete (Figure 1.1, Bartholomew, 1997). In the fish host, hemorrhaging and necrosis of the intestinal tissues may occur from the infection, resulting in the disease ceratomyxosis (Bartholomew, 1998).

Ceratomyxa shasta is native to the Pacific Northwest region of North America, although the parasite life cycle has not established in many coastal rivers and tributaries. This parasite has been identified from rivers in British Columbia (Fraser), Oregon (Klamath, Willamette, Deschutes, Rogue and Columbia (also in WA and ID)), California (Sacramento, San Jaoquin, Klamath) and areas of Washington, Idaho and Alaska (Schafer, 1968; Johnson, 1975; Udey et al., 1975; Johnson et al., 1979; Ratliff, 1983; Ching and Munday, 1984a; Hendrickson et al.,1989; Hoffmaster et al., 1988; Bartholomew et al., 1989a; Ted Meyers, ADFW, pers. comm.). Parasite distribution is...
seasonal; infection in fish generally occurs from late spring until late fall to early winter, with a peak in infection from May to June. The parasite can be detected when temperatures range from 4-23°C, and infections in fish usually occur when temperatures exceed 10°C (Schafer, 1968; Johnson, 1975; Udey et al., 1975; Johnson et al., 1979; Ratliff, 1983; Ching and Munday, 1984a; Hendrickson et al., 1989).

Recent genetic analyses have demonstrated distinct genotypes of the parasite. Genetic information on myxozoans is minimal and no myxozoan genomes have been sequenced to date, limiting the development of molecular techniques. Consequently, genetic analyses of the ribosomal gene operon, including the small subunit rRNA, internal transcribed spacer region 1 (ITS-1), 5.8S, ITS-2, and large subunit rRNA were conducted using an established primer for Ceratomyxa shasta and a general myxozoan primer (Palenzuela et al., 1999; Atkinson and Bartholomew, 2010a). Analyses of the more variable internal transcribed spacer region I (ITS-I) of the parasite genome identified a characteristic trinucleotide repeat region that differentiated between four parasite genotypes 0, I, II and III.

THE HOSTS

Salmonid susceptibility to the parasite differs by fish species and also within strains of the same species (Zinn et al., 1977). Fish strains of the same species that are sympatric with the parasite have a lower susceptibility to the parasite. Parallel exposures of six hatchery strains of Chinook salmon, three strains endemic to the Columbia River and three from coastal rivers, to C. shasta in the Willamette River highlight differences in susceptibility based on fish origin. Zinn et al. (1977) demonstrated that mortality was higher in stocks from coastal rivers where the parasite is allopatric with the host. Similarly, Buchannan et al. (1983) showed that an Oregon coastal strain of steelhead trout was highly susceptible to the parasite, whereas three Columbia River strains displayed low susceptibility. However, parasite-induced mortality was similar among Chinook strains from the Fraser River regardless of whether the fish had prior historic exposure, suggesting additional variables influence fish susceptibility (Ching and Parker, 1989, see Bartholomew, 1998).
The likelihood of a fish developing ceratomyxosis also depends on parasite dose, which is affected by exposure duration, and water velocity and temperature, of which the latter influences parasite replication rate in the host. Researchers showed that prolonged exposure to the parasite could overwhelm the inherent resistance of the fish host, regardless of the susceptibility level of the fish (Zinn et al., 1977; Ratliff 1981). Research in our laboratory has demonstrated that high parasite doses could cause disease in low susceptibility strains of fish (Bjork and Bartholomew, 2010; Ray et al., 2010). In addition, water flow can indirectly influence parasite dose, as fish exposed to slower flows had a faster mean day to death, indicating exposure to a higher parasite dose (Bjork and Bartholomew, 2009). Once the parasite has infected the fish, temperature affects the progression of the disease, with increased temperatures resulting in a faster mean day to death (Udey et al., 1975). Disease in fish becomes apparent as water temperatures warm to 10°C, although infection has been documented in exposures with temperatures as cool as 4-6°C (Schafer, 1968; Ratliff, 1983; Ching and Munday, 1984b). Temperature may also influence the progress of ceratomyxosis in different species of fish. Rainbow trout exposed to the parasite had a reduced mean time to death at warmer temperatures and disease was suppressed below 4°C. Coho salmon were better able to resist disease if they were maintained at lower temperatures, but still succumbed to infection at warmer temperatures (Udey et al., 1975).

The recent elucidation of four parasite genotypes also affects our understanding of fish susceptibility, as parasite genotypes 0, I and II were associated with different salmonid hosts. By determining the parasite genotype of hundreds of fish from different species and different areas in the Klamath River, Atkinson and Bartholomew (2010a, b) demonstrated that genotype II predominated in rainbow trout and coho salmon. Genotype 0 was isolated predominantly from steelhead and Klamath redband trout and genotype I was isolated only from Chinook salmon. Genotype III was never identified as a dominant genotype in fish and consequently was not associated with any particular salmonid host. Thus, all studies previously conducted with C. shasta, including host susceptibility, dose,
exposure period and temperature should be re-analyzed to incorporate parasite genotype information.

Far less research has concentrated on the polychaete host for *Ceratomyxa shasta*. Populations of the polychaete have been identified throughout the Klamath River basin, mostly associated with fine benthic organic matter, *Cladaphora spp.*, and attached to boulders within runs, riffles and eddies (Stocking and Bartholomew, 2007). Stocking and Bartholomew (2007) found that 13 populations of polychaetes had densities ranging from less than 1,000 to in the tens of thousands. Even in large populations (> 16,000), such as the population at Tree of Heaven below Iron Gate Dam, infection prevalence remained low (0-8%). Therefore, it appeared that large populations of polychaetes with low infection prevalence are sufficient for 100% mortality of allopatric rainbow trout. Similar to the effects that water velocity has on infections in fish, infection prevalence in polychaetes was influenced by flow dynamics. Bjork and Bartholomew (2009) demonstrated that flow significantly influenced disease dynamics in laboratory polychaete cultures. In fast flows (0.05 m s\(^{-1}\)) polychaete densities were higher, but infection prevalence was lower than in cultures maintained with slow flows (0.01 m s\(^{-1}\)). Stocking and Bartholomew (2007) showed that water velocities of 0.02-0.05 m s\(^{-1}\) were ideal for natural polychaete populations.

**CERATOMYXA SHASTA IN THE KLAMATH RIVER SYSTEM**

The Klamath River Basin in Oregon and California is 480 Rkm, beginning with the headwaters in the Williamson River (WMR), Oregon and ending with the outflow into the Pacific Ocean in northern California (Figure 1.2). A series of five dams constructed between 1917 and 1962 has divided the basin into upper and lower portions (Figure 1.2). *C. shasta* has been identified throughout the Klamath River, but disease dynamics differ between the upper and lower basin (Hallett and Bartholomew, 2006; Stocking et al., 2006). One reason for the difference in disease dynamics is the discontinued migration of anadromous salmonids into the upper basin following construction of Iron Gate Dam. The lack of anadromous fish in the upper basin has led to a disparity in parasite genotypes between the upper and lower Klamath basin (Atkinson
and Bartholomew, 2010a). In the lower basin, the presence of all four parasite genotypes overlaps with the presence of their respective hosts. However, the absence of genotype I from the upper basin demonstrates how parasite genotype populations may have changed since the extirpation of these fish.

With the signing of the Klamath Basin Restoration Agreement (2010), four of the five dams are slated for removal beginning in 2020. In addition, fish passage facilities will be constructed at Keno Dam, allowing anadromous salmonids to repopulate their historic ranges. Anadromous salmonids present in the upper basin before the construction of the dams included spring Chinook and coho salmon and steelhead trout. The range of Chinook salmon in the upper basin extended to the falls below Klamath Marsh and into the Sprague River (Spier, 1930; Fortune et al., 1966; Lane and Lane Associates, 1981 see Hamilton et al., 2005). The habitat ranges of steelhead and Chinook salmon usually overlap, with steelhead often having a wider range (Hamilton et al., 2005). Therefore, it is likely that steelhead utilized habitat as far upstream as the Williamson and Sprague Rivers (Fortune et al., 1966 see Hamilton et al., 2005). The historical distribution of coho salmon in the upper Klamath basin was at least as far upstream as Spencer Creek and may have extended further (Hamilton et al., 2005).

The pending reintroduction of these fish into the upper Klamath basin, where C. shasta is present in high densities in the WMR, raises concerns about the health of reintroduced and native upper basin fish strains once these isolated populations come into contact. Since 2004, the Bartholomew laboratory has conducted caged (sentinel) fish studies to monitor changes in parasite distribution and fish mortality rates. The use of sympatric strains of Chinook and coho salmon highlights areas that affect native Klamath salmonid species, namely in the Klamath River below Iron Gate Dam (Stocking et al., 2006). In addition, allopatric rainbow trout (RBT) are utilized to detect the parasite at densities below the native Klamath fish threshold of infection (Bjork and Bartholomew, 2010; Ratliff, 1983). These studies show that the parasite is distributed throughout the main-stem Klamath River, but not in any tributaries apart from the Williamson River.
To minimize the use of endangered and threatened fish strains in sentinel studies, a quantitative polymerase chain reaction assay (qPCR) was developed to analyze parasite density and distribution patterns in Klamath River water samples throughout the year (Hallett and Bartholomew, 2006). These monitoring studies have demonstrated that densities of parasite measured in the WMR were similar (>10 parasites L\(^{-1}\)) to those in the Klamath basin below Iron Gate Dam, despite the lack of anadromous fish. The high densities of parasites in the WMR as compared to other upper basin locations and the importance of this river in the anadromous fish reintroduction plan highlight the need for further study in the WMR drainage.

The headwaters for the Williamson River stem from cool springs located in the Winema National Forest. Before reaching the first major tributary, Spring Creek, the WMR travels about 80 Rkm, likely warming in temperature as the river passes through Klamath Marsh. The Spring Creek tributary is predominately a cool spring-fed tributary that provides cool water inputs year-round into the WMR (Figure 1.2). In contrast, inputs from the Sprague River tributary are warmer than Spring Creek despite being dominated by snow melt in the upper region and springs near the WMR confluence (Ganette et al., 2010). Warmer temperatures in the Sprague are likely an indirect result of the extensive irrigation and agriculture in the Sprague River Basin. Land-use practices in this region have cleared away much of the riparian habitat that historically shaded the river and maintained cooler river temperatures (Boyd et al., 2001 see Committee and NRC, 2004). Together the Williamson and Sprague Rivers provide over half of the water for upper Klamath Lake (Kann and Walker, 2001 see Committee and NRC, 2004).

Despite the exclusion of anadromous fish from the upper basin, salmonids do inhabit the WMR drainage. Native salmonids currently present in this system include redband trout, bull trout (\textit{Salvelinus confluentus}) and kokanee salmon (resident form of sockeye; Logan and Markle, 1993 see Hamilton et al., 2005). Behnke (1992 see Committee and NRC, 2004) states that two distinct subspecies of redband trout exist in the upper basin, the lake form and one that resides in isolated streams, although the Oregon Department of Fish and Wildlife recognizes only one subspecies. Introduced
salmonids into the Klamath River basin include brook trout, brown trout and rainbow trout. Rainbow trout (Cape Cod 72 strain) have been released into the Spring Creek tributary of the WMR since 1925 to supplement recreational fishing. The rationale for using this strain of fish was that their high susceptibility to the parasite results in ceratomyxosis shortly after their release, thus preventing this fish strain from interbreeding with the native redband trout (W. Tinniswood, ODFW, pers. comm.). However, Bowers and colleagues (1999 see Committee and NRC, 2004) stated that some interbreeding with native redband trout had been noted.

Studies on *C. shasta* in the upper basin since the 1980’s have concentrated predominately in the WMR and have utilized both allopatric and sympatric fish strains (Hemmingsen *et al.*, 1988, Buchannan *et al.*, 1989, Stocking *et al.*, 2006). In the 1980’s, researchers showed that the highest mortality (90-100%) in rainbow trout occurred at WMR river kilometer (Rkm) 3.2 and 19 (Sprague River confluence; Hemmingsen *et al.*, 1988; Buchannan *et al.*, 1989). Buchannan and colleagues (1989) hypothesized that since mortality of allopatric rainbow trout did not occur in Spring Creek, Spencer Creek, or Rkm 360 of the main-stem Klamath River, the parasite was absent from these areas. An alternative hypothesis is that the genotype present in these areas is not the genotype (II) that causes ceratomyxosis in rainbow trout. In exposures of allopatric rainbow trout at Rkm 368, Atkinson and Bartholomew (2010a) only identified genotype 0 from both water and fish. Thus, the presence of only genotype 0 could explain the survival in rainbow trout at Rkm 360, Spencer Creek and Spring Creek.

Streams in the upper basin support genetically distinct populations of redband trout (Reiser *et al.*, 2001 see Committee and NRC, 2004). Mortality of sympatric redband trout collected from various creeks in the upper basin and exposed in the WMR at Rkm 3.2 ranged from 0-100% (Buchanan *et al.*, 1989). No mortality occurred in redband populations collected from Spring Creek, Spencer Creek or the main-stem Klamath River, similar to the survival of allopatric rainbow trout exposures observed in these regions. The absence of mortality in redband populations native to these creeks suggests
these populations migrate into the main-stem Klamath River and the WMR, where they are exposed to the parasite and gain resistance (Buchanan et al., 1989).

Sentinel exposures of fall Chinook salmon in the WMR and in upper Klamath Lake have resulted in low mortality and infection prevalence. In one study conducted in 2003 and 2004 exposure of Iron Gate fall Chinook salmon in the WMR resulted in no infection or mortality (Stocking et al., 2006). Another study designed to assess the feasibility of Iron Gate fall Chinook salmon as a candidate for anadromous fish reintroduction into the upper basin, exposed this Chinook salmon strain in Upper Klamath Lake, the WMR, and Beaver Creek, the latter a section of the lower Klamath River (Maule et al., 2009). Although about 20% mortality and 45% infection prevalence in Chinook occurred at Beaver Creek, no parasite-induced mortality resulted from exposures at any upper basin sites, likely because genotype I has not been identified in the upper basin (Atkinson and Bartholomew, 2010a).

*Ceratomyxa shasta* disease dynamics throughout the Klamath River basin are of concern for reintroduced anadromous salmonids. Returning adults likely become infected in the lower river and could carry lower basin genotypes into the upper basin. The high densities of parasite currently in the lower WMR and the identification of a number of polychaete populations in the upper basin suggest that establishment of lower basin genotypes is likely. In addition, the high parasite densities could also pose problems for returning adults and migrating juveniles. Although no Chinook mortality has been observed in sentinel exposures in the upper basin, likely because genotype I is absent, genotype II is present and could adversely affect returning coho. Thus, more research about parasite dynamics in the upper basin, especially the WMR, is necessary for predicting any adverse affects on anadromous salmonid reintroduction into the upper Klamath basin.

**GOALS AND OBJECTIVES**

The overall goal for this thesis was to determine how current *C. shasta* disease dynamics in the upper basin, and particularly in the WMR, will affect the reintroduction of
anadromous salmonids into the upper Klamath basin. To address this goal, I had the following objectives:

- Expand on the known parasite distribution and determine parasite density and genotype composition in the WMR.
- Assess the influence of stocking non-native rainbow trout on parasite density and genotype composition in the WMR.
- Ascertain the virulence of parasite genotypes I and II in allopatric rainbow trout and Chinook salmon and sympatric (Iron Gate) strains of fall Chinook and coho salmon.
1.1 *Ceratomyxa shasta* life cycle.
1.2 The Klamath River Basin with the Williamson River outlined and enlarged to the right
CHAPTER 2: CERATOMYXA SHASTA DYNAMICS IN THE WILLIAMSON RIVER, OREGON: IMPLICATIONS FOR REINTRODUCED ANADROMOUS SALMONIDS

Charlene N. Hurst, Richard A. Holt and Jerri L. Bartholomew
ABSTRACT

*Ceratomyxa shasta*, a myxozoan parasite of salmonids, occurs throughout the Klamath River (KR), Oregon/California, USA. Parasite densities are as high in the Williamson River tributary (WMR) of the upper Klamath basin as those in the lower basin, where high native fish mortality occurs (Foot et al., 2004), raising concerns over the success of plans to reintroduce anadromous fish into the upper basin. In the WMR, parasite density, distribution, genotype composition and host overlap were examined to determine how reintroduced fish might be affected by disease dynamics. Parasite density in water samples collected throughout the WMR and its tributaries was determined using a *C. shasta*-specific quantitative PCR assay. High parasite densities occurred in two reaches of the river: between the mouth of the WMR and the confluence of Sprague River (river kilometer [Rkm] 19) and above the Spring Creek confluence (Rkm 27.5) to Rkm 33. A survey for the invertebrate host identified one assemblage in each area of high parasite density, with the larger of the two assemblages detected in the lower river. Genetic analyses of parasites from water samples and infected fish in the WMR demonstrated that the dominant parasite genotype was associated with mortality of the stocked non-native rainbow trout. Comparison of parasite density, genotype and mortality of infected sentinel (caged) fish held in the WMR and at a site in the mid-Klamath River where no stocking occurs, Keno Eddy, suggested that stocking non-native fish may have resulted in amplification of the parasite. The lack of coho or Chinook mortality at either site demonstrated that despite high parasite densities, the genotypes associated with these native salmonids are not present. However, returning adults will introduce lower basin genotypes that are likely to become established as conditions in the WMR are conducive for the completion of the *C. shasta* life cycle.

Keywords: Myxozoa, Klamath River, salmonid, genotype, reintroduction
INTRODUCTION

Anadromous salmonids require both freshwater and marine environments for the completion of their life history. Freshwater systems like the Klamath River (KR), which extends 480 river kilometers (Rkm) from the Williamson River (WMR) in Oregon to the Pacific Ocean in California, provide critical spawning habitat for anadromous fishes. However, five dams separate the upper and lower KR basin and anadromous fish have not had access to the upper basin since the construction of the Copco I Dam in 1917. With anadromous salmonid populations declining in the lower KR basin, the need for additional spawning habitat is paramount. The Klamath Basin Restoration Agreement and Klamath Hydrologic Settlement Agreement (2010) provide for increased habitat in the upper KR basin through the removal of the four lower dams and fish passage facilities at the fifth dam (Keno). Historically, the ranges of spring Chinook salmon (Oncorhynchus tshawytscha), coho salmon (O. kisutch) and steelhead (O. mykiss, anadromous form) extended into the upper basin (Hamilton et al., 2005) and after conditions in the agreement are met, these fish are expected to repopulate their historic ranges.

The success of anadromous salmonid reintroduction is contingent on their ability to cope with pathogens in the KR basin. One major fish health concern is the salmonid parasite, Ceratomyxa shasta. This parasite is endemic to the Klamath River basin and causes ceratomyxosis, a disease of salmon and trout characterized by severe inflammation, hemorrhage and necrosis of the intestine (Bartholomew et al., 1989). Infection often results in death, although fish native to rivers where the parasite is endemic are less likely to develop the disease (Zinn et al., 1977; Bartholomew, 1998). This parasite has a complex life cycle that requires a salmonid host and polychaete worm, Manayunkia speciosa. The polychaete releases the actinospore stage which infects the salmonid and the salmonid releases the myxospore stage, infecting the polychaete (Bartholomew et al., 1997). Parasite distribution is seasonal, with infection occurring in sentinel fish exposed between April and mid-December, usually at temperatures above 10°C (Ratliff, 1983; Ching and Munday, 1984a; Hendrickson et al., 1989). The
seasonality of the parasite coincides with the returns of adult spring Chinook, the fish strain most likely to utilize upper KR basin habitat (Hamilton et al., 2005).

In the upper Klamath basin, high densities of both the parasite and polychaete host have been detected in the WMR. From the mouth of the WMR to the Sprague River confluence (Rkm 19) 90% parasite-induced mortality has been observed in introduced rainbow trout (*O. mykiss*, freshwater form) since the 1980’s (Hemmingsen et al., 1988; Buchanan et al., 1989; Hallett and Bartholomew, 2006; Stocking et al., 2006). Assays of water samples collected during sentinel studies in 2004 demonstrated that parasite densities in the WMR were similar to those in the lower KR (Hallett and Bartholomew, 2006). In addition, a survey for the polychaete host identified a large population at the WMR confluence with Klamath Lake (Stocking and Bartholomew, 2007). These studies demonstrated that appropriate conditions exist for *C. shasta* to persist in the WMR at high densities and cause high mortality in introduced rainbow trout.

There are four genetically distinct genotypes of *C. shasta* (0, I, II, and III) that occur in sympatry with their salmonid hosts in the KR basin (Atkinson and Bartholomew, 2010a, b). Thus, the ability of the parasite to establish and reproduce is influenced not only by the numbers, but also by the species and strain of salmonid hosts available. Since 1925 non-native rainbow trout have been stocked into the Spring Creek tributary of the WMR to supplement recreational fishing. Fish stocking occurs during the peak months for parasite production, from May through August, and because of their high susceptibility to *C. shasta*, these fish are assumed to die of ceratomyxosis from genotype II (Atkinson and Bartholomew, 2010a) if not caught by anglers (W. Tinniswood, ODFW, pers. comm.). This strategy ensures that these fish will succumb to ceratomyxosis before interbreeding with native redband trout (*O. mykiss newberrii*), but may also amplify certain parasite genotypes in the WMR.

This study expands our understanding of *C. shasta* in the WMR by describing its distribution and density and by characterizing the genotype composition of the parasite from water samples. To understand how stocking practices may alter parasite dynamics, parasite genotype composition, parasite density and mortality of native and introduced
sentinel fish were compared between the WMR (sWMR) and a site downstream in the main-stem KR at Keno Eddy (sKED) where stocking does not occur. Understanding how biotic factors such as fish and polychaete hosts, abiotic factors such as temperature and management practices influence the distribution, density and genotype composition of *C. shasta* is important for predicting how the current parasite dynamics will affect reintroduced anadromous salmonids in the upper Klamath basin.

**METHODS**

I. *Ceratomyxa shasta* density, distribution and genotype composition in the Williamson River

*Water sampling:* Four x 1L samples of water were collected from 20 sites between Rkm 0 and 42 (Figure 2.1) in September 2008, June 2009 and May and June 2010. The main-stem river was divided into three reaches based on geomorphology. Reach 1 comprised the area from the mouth of the Williamson River to the Sprague River confluence, Reach 2 included the area between Sprague River and Spring Creek and Reach 3 consisted of the area above Spring Creek to Rkm 42. Sites were selected based on accessibility and representation of the river, with sites in all three reaches and two sites within each tributary. Sampling occurred during sentinel fish exposures (see below). Mercury thermometers were placed ~six inches below the surface and GPS latitudes and longitudes were recorded for each site using a Garmin GPS 72 (Olathe, KS). Sites were the same at all time points with the exception of Rkm 36 in June of 2009 to replace sites at Rkm 36.3 and 36.6 as high water levels prevented access. All samples were kept on ice during transport to the John L. Fryer Salmon Disease Laboratory, Oregon State University, Corvallis Oregon, USA (SDL). Water was filtered 24 to 48 hours after collection and the filter and its retentate were frozen at -20°C until processed (Hallett and Bartholomew, 2006). The processing procedure was modified in 2010 to use acetone to dissolve the filter membrane (versus cutting). DNA was extracted from three samples from each site and each sample was assayed in duplicate by quantitative polymerase chain reaction (qPCR, Hallett and Bartholomew, 2006) along with negative (molecular grade water) and positive controls (*C. shasta* infected tissue), using an ABI7300 qPCR.
sequence detection system. Inter assay variability was assessed from the standard deviations of the positive control samples. Quantitative cycle threshold (Cq) values for three site replicates were averaged and tests for inhibition were conducted on one sample from each site (Hallett and Bartholomew, 2009). Samples were considered inhibited if a difference greater than one cycle occurred between duplicate runs of the same sample. Inhibited samples were re-analyzed with a 1:10 dilution of parasite DNA to molecular grade water. Parasites L$^{-1}$ was determined based on a one spore standard reference sample. Sites with < 1 parasite L$^{-1}$ were considered negative. Parasite density threshold values of 10 parasites L$^{-1}$ and 100 parasites L$^{-1}$ were extrapolated from the one spore standard and on previous correlations (Hallett and Bartholomew, 2006).

**Spore Stage Identification:** To determine the spore stage at locations with water samples that tested positive (the qPCR assay does not differentiate between spore stages), 5L of water were collected at Rkm 18.2 and 33 in June of 2009, in conjunction with the rest of the WMR water sampling (Figure 2.1). Sites were selected based on results from water samples collected in September 2008. Samples were transported on ice to the SDL and water was poured into separate 25L fiberglass tanks with airstones. Ten rainbow trout (RBT, Roaring River Hatchery, OR) were placed in each tank. After 72 hours of exposure, flow into each tank was resumed with pathogen-free water and fish were monitored daily for signs of disease. Moribund fish were euthanized using tricaine methanesulfonate (MS-222, Argent laboratories) and a swab of the intestinal tissue was examined under a compound microscope at 200X for the presence of myxospores. Intestinal tissues were collected for sequencing of the parasite DNA (see below) Surviving fish at 60 days post exposure were euthanized and processed as above.

**Polychaete Density and Infection Prevalence:** Sites surveyed for polychaetes were selected based on water sample data (parasite densities of > 1 parasites L$^{-1}$ within the site and no parasite detected above the site which would indicate infected host populations) and suitable polychaete habitat (fine benthic organic matter, boulders, or *Cladaphora spp.*; Stocking and Bartholomew, 2007). Two polychaete sampling sites fit these criteria: from Rkm 18.2 to 19.2, which straddles the Sprague confluence (Reaches 2 and 3); and
above Spring Creek (Rkm 33 to 34 within reach 3). To focus our search efforts within each polychaete site, 2 x 1L water samples were collected ~every 0.1 Rkm and processed using qPCR as described above. One 0.1 Rkm segment within Rkm 33 to 34 and two segments within Rkm 18.2 to 19.2 were identified with parasite densities > 1 parasite L\(^{-1}\). Ten samples were collected from the segment within Rkm 33 to 34 and 7 samples from the two segments within Rkm 18.2 to 19.2 on July 20-21, 2010. When water levels were deep, two divers with SCUBA collected benthic invertebrates. A Scotch brite® scrub pad was used to dislodge benthic invertebrates from substrate contained within a Hess Sampler and were then collected in the Sampler’s 80µm aquatic mesh. Samples were transferred to Whirl-Pak® plastic bags and immediately preserved with 95% ethanol. Samples were then placed on ice for transport to the SDL.

In the laboratory, samples were emptied into a 30 x 20cm\(^2\) 180-D10 tray (Wildco, Yulee, FL) subdivided into 15 sections. Three 5cm\(^2\) subsamples were randomly selected and material within each section was collected using a disposable pipette and placed in 5mL glass vials. A 1:3 ratio of Rose Bengal dye (20mg L\(^{-1}\) 95% ethanol) to 95% ethanol was added to the vial prior to sorting. After 24 hours, the sample was filtered through an 80µm sieve to remove dye and then stored in 95% ethanol. Polychaetes were sorted using a dissecting microscope at 100x magnification. This procedure was repeated for all three subsamples. Individual polychaetes were placed into 1.5mL microcentrifuge tubes with ~100µL of 95% ethanol and refrigerated until processing. Polychaete densities were calculated by averaging the three subsamples and extrapolating to polychaetes m\(^{-2}\).

DNA from individual polychaetes was extracted according to Stocking and Bartholomew (2007) with the following modifications: DNA extraction buffer was reduced to 100 µL, Proteinase K was reduced to 5µL, and following addition of RNase, sample incubation at 37°C was extended to one hour. After boiling, pools of five polychaetes were created by adding 2µL of each sample into a tube (10µL total), then adding 2µL of the pooled DNA into 198 µL of distilled water. Pooled DNA was assayed for *C. shasta* by qPCR (Hallett and Bartholomew, 2006). Polychaetes from positive pools
were then assayed individually by qPCR using a 1:100 dilution of the original sample to determine infection prevalence.

**Sequencing:** *Ceratomyxa shasta* DNA was extracted from water samples, polychaetes and fish intestinal tissues as above. One µl of the final eluate for water samples, 1µl of a 1:100 dilution of the fish samples and 2µL of a 1:100 dilution of polychaete samples was used in the genotyping polymerase chain reaction assay (PCR, Atkinson and Bartholomew, 2010a). Samples were purified using Exosapit (USB, Cleveland, OH) and submitted to the Center for Genomics and Bioinformatics at Oregon State University for sequencing using an ABI Prism® 3100 Genetic Analyzer. Parasite genotype was determined according to the number of trinucleotide repeats in the internal transcribed spacer region I and genotype proportions in mixed samples were identified by comparing chromatogram peak heights (Atkinson and Bartholomew, 2010a).

**Statistical Analyses:** S-plus statistical software version 8.1.1 (Tibco, Palo Alto, CA) was used to perform all statistical analyses. Temperature and average Cq-values per site between river reaches (1, 2 and 3) and Cq values between months were compared using a one-way ANOVA. To determine which river reach and month were significantly different, a Tukey test was performed. Differences were considered significant when p-values were < 0.05. A non-linear regression was conducted with temperature versus average Cq-values over all time and sites.

**II. Sentinel Fish Studies in the Upper Basin**

**Fish Exposures:** Sentinel fish exposed as part of a comprehensive parasite monitoring study from 2007 to 2010 were used to assess differences in RBT mortality between the WMR (sWMR, 42°30.820N, 121°55.021W), where stocking of RBT occurs and an area of the upper basin without RBT stocking at Keno Eddy (sKED, 42° 8.9835N, 122°0.9332W). In 2009, the sWMR site was moved further downstream for accessibility reasons (42°29.661N, 121°56.095W, Figure 2.1). Rainbow trout (Roaring River Hatchery, Scio, OR) were held at all exposures. The use of Chinook and coho salmon illustrated effects of upper basin parasite dynamics on anadromous fish strains. Fall Chinook salmon from Iron Gate (IG) Hatchery (Hornbrook, CA) were exposed at sWMR
from 2006 to 2010. Iron Gate coho salmon exposures were conducted at sWMR in May of 2007 and in May and June of 2009. Fish exposures and subsequent monitoring from 2006 to 2010 followed methods in Stocking et al. (2006).

**Water Sampling:** To assess parasite density during sentinel fish exposures, 3 x 1L samples of water were taken at the beginning and end of each fish exposure at each site, except in June 2008, when water samples were only collected at the beginning of the exposures. Water samples were processed and assayed using qPCR, as above.

**Sequencing:** To determine if parasite genotype differed between the two upper basin sentinel sites, parasite DNA was sequenced from ten *C. shasta* positive RBT exposed in June over all years. When possible, fish were selected from both the mortality and survivor groups for parasite genotype comparison. Sequencing was performed as detailed above.

**Statistical Analyses:** Average Cq values and percent mortality differences between sites, sWMR and sKED, and months, May and June, were compared using two-sample t-tests in S-plus version 8.1.1 (Tibco, Palo Alto, CA). Differences were considered statistically significant when p-values were < 0.05.

**RESULTS**

I. *Ceratomyxa shasta* Density and Distribution in the Williamson River

Parasite density was not significantly different between months (one-way ANOVA, F(3, 58 = 1.59, p = 0.20) but did differ when analyzed by reach (one-way ANOVA, F (2.59) = 52.2, p< 0.001). Reach 1 parasite densities were significantly different (Tukey, 2.40) from both Reaches 2 and 3, with parasite density > 10 parasites L\(^{-1}\) at all sites in reach 1. Parasite densities in Reach 2 between the tributaries were < 1 parasite L\(^{-1}\), except for Rkm 19.2 in June 2009 where parasite densities exceeded 1 parasite L\(^{-1}\). In Reach 3, two sites consistently had parasite densities of 1-10 parasites L\(^{-1}\): just above Spring Creek at Rkm 28 and Rkm 33 (Figure 2.2). The confluences with the WMR tributaries, Spring Creek and Sprague River occur at Rkm 27.5 and Rkm 19, respectively, and in both tributaries parasite densities were < 1 parasite L\(^{-1}\) during all
sampling times (Figure 2.2). Variability between qPCR assays was low, with standard deviations of 1.1 and 0.9 cycles for both reference samples.

Water temperatures varied by sampling month and river reach, but were warmer in Reaches 1 and 3 than 2 (one-way ANOVA, F (2, 58)=8.43 , p < 0.001, Tukey 2.41). Comparison of main-stem river temperatures between months demonstrated temperatures were higher in June than in September and May (one-way ANOVA, F (3, 58) = 13.9, p <0.001, Tukey 2.65). However, a wider temperature range was observed in June (5-22°C), than in September and May (8-16°C). Temperatures in Reach 2 ranged from 5-14°C, while temperatures in Reaches 1 and 3 were similar and ranged from 10-21°C and 8-22°C, respectively. Temperatures in the Sprague River were consistently warmer than in the WMR during all months, with temperatures in June of 23°C and in May and September of 16°C. Spring Creek maintained a relatively constant cool temperature of 5-8°C regardless of month. A polynomial regression \( y = 0.0641x^2 - 2.1108x + 50.144, R^2 = 0.1976 \) showed that about 20% of the variability in parasite density was explained by temperature, and demonstrated that parasite production occurred when the temperature was between 10°C and 21°C (Figure 2.3).

Two of the four known genotypes were detected in the WMR. The proportions of parasite genotypes II and 0 in water samples changed spatially and temporally, with larger proportions of genotype 0 detected in Reach 1 than Reach 3. Genotype 0 accounted for 10-30% of the parasites present in water samples in Reach 1 at all sampling points.

One exception occurred in May 2010, when water collected from Rkm 18.2 contained 82% genotype 0. This was the only time when the proportion of genotype 0 was greater than genotype II. Genotype 0 was not detected at any sites in Reach 3, except in June 2009 when it comprised approximately 10% (Figure 2.4). Parasite densities in Reach 2 were below the sequencing threshold, except in June 2009 when genotype II accounted for 100% of the parasites detected.

Infection occurred in RBT exposed for identification of parasite spore stages in water collected from either Reach 1 (100% prevalence and mortality) or Reach 3 (20%). All fish were infected with genotype II; genotype 0 was not detected.
One polychaete assemblage was identified at Rkm 18.3 in Reach 1 and one population at Rkm 33.2 in Reach 3. Polychaete density below the Sprague River was calculated as 40,765 polychaetes m$^{-2}$ and infection prevalence was 1.1% (n=185). Only genotype II was detected in the polychaetes. In Reach 3, polychaete density was estimated at 78 polychaetes m$^{-2}$ and infection prevalence was 0% (n=2).

**II. Sentinel Fish Studies in the Upper Basin**

Differences in RBT mortality and parasite density were evident between sWMR and sKED (Figure 2.5), but mortality of IG Chinook and coho salmon was less than 5% at both sites. From 2006 to 2010, mortality in RBT trout held at the sWMR site at all exposures was nearly 100%, while mortality at sKED ranged from 0 to 75%. Mortality comparisons between sites for RBT were statistically significant for all exposures (2 sample t-test, t (18) = -9.7, p < 0.001). Parasite density differences between sites were also statistically significant; density at sKED averaged < 1 parasite L$^{-1}$, 10-100 fold less than at sWMR, where density averaged >10 parasites L$^{-1}$ (2 sample t-test, t (24) = 7.7, p < 0.001).

Parasite genotype differed between RBT at the two locations. All mortalities from the sWMR site were infected with genotype II. RBT mortalities from sKED were infected with either genotype 0 (50%) or genotype II (50%) and survivors were infected with genotype 0.

**DISCUSSION**

*Ceratomyxa shasta* density, distribution and genotype composition within the Williamson River (WMR) are influenced by many factors: water temperature, fish and polychaete hosts and management practices. Our study identified high *C. shasta* densities from the mouth of the WMR to the Sprague River confluence, no parasite detection between the tributaries, and low parasite densities from the Spring Creek confluence to 6 Rkm above. The distribution of the parasite likely reflects water temperature, location of polychaete populations and movement of the fish hosts, particularly the introduced RBT. Parasite genotype composition differed between the upper and lower portions of the
WMR as well as between exposure locations in the lower WMR and Keno Eddy (sKED), an upper basin site between the J.C. Boyle and Keno Dams.

The WMR is predominately a spring-fed system with inputs from two major tributaries. The resulting temperature patterns affect parasite density and distribution. Throughout the WMR, the parasite was detected at locations where water temperatures were between 10 and 21˚C. Water temperatures in the lower WMR (Reach 1) are influenced by the Sprague River. Although water sources for the Sprague include a combination of snowmelt and cool springs, land use practices in this drainage have likely decreased natural riparian shading, resulting in warmer temperatures (Boyd et al., 2001 see Committee and NRC, 2004). Thus, in the WMR below Sprague River, temperatures rise above 10˚C in April, peak in July, then continue to decline, falling below 10˚C in October (USGS, http://waterdata.usgs.gov/or/nwis/rt; this study). Consequently, parasite densities below the Sprague River confluence were high during all sample times (May, June and September).

Above the Sprague River confluence, in Reach 2, temperatures were largely influenced by cool springs in Spring Creek (Ganette et al., 2010), which is a constant source of cold water (5-8˚C) even during the summer months. Although both hosts may be present, temperatures are below the threshold of both actinospore production and fish infection (Schafer, 1968; Johnson et al., 1975; Udey et al., 1975; Ratliff, 1983; Hendrickson et al., 1989; Bjork, 2010). Therefore, the cool water temperature between Spring Creek and Sprague River likely limits parasite production.

Parasites were detected in the WMR above Spring Creek (Reach 3) for 6 Rkm, but were not detected further upstream despite temperatures similar to those below the Sprague River confluence. The upper portion of Reach 3 at Rkm 42 is ephemeral, with no flow from late summer through winter (USGS, http://waterdata.usgs.gov/or/nwis/rt) and likely reduced flows further downstream where polychaetes were identified. This flow variability may naturally constrain polychaete populations, which require a certain amount of flow for filter feeding and to prevent desiccation (Stocking and Bartholomew, 2007; Bjork and Bartholomew, 2009). Thus, water temperatures may limit the
distribution of the parasite between the Sprague River confluence and Spring Creek, with water flow restricting parasite range in the upper reaches of the WMR.

Water temperature also affects the distribution of trout in the WMR and the Sprague River, thereby limiting parasite distribution to areas of the system where infected fish occur. Stocking of RBT occurs from late May to late August (W. Tinniswood, pers. comm.), when temperatures are optimal for the parasite. However, the migration of non-native RBT into the Sprague River may be discouraged by the warmer water temperatures (Cherry et al., 1976) in that tributary (5-7°C warmer) compared with the main-stem WMR. When water temperatures cool in the Sprague River to within the optimal temperature ranges for the fish and parasite in September, RBT have likely already died of ceratomyxosis. Thus, the absence of susceptible fish in the Sprague River during times when environmental conditions are most favorable for the parasite may explain why we were unable to detect *C. shasta* in this tributary.

The distribution and density of the different parasite genotypes provides some evidence as to where fish move in the system. Native redband trout become infected with *C. shasta* (Buchanan et al., 1989), however their low susceptibility suggests that they release fewer parasites compared to the high susceptibility RBT. Native redband trout become infected with genotype 0 (Atkinson and Bartholomew, 2010b), which only accounted for 20-30% of the total parasite detected in water below the Sprague River confluence and an even smaller proportion of parasites above Spring Creek. The smaller proportions of genotype 0 compared to genotype II, and the low susceptibility of native fish to the parasite, suggest that native redband trout are not responsible for the high density of parasites in the WMR.

Comparisons of fish mortality, parasite density and parasite genotype between the WMR and sKED demonstrated a difference in parasite dynamics. Parasite genotypes differed between the WMR and sKED, with genotype II predominant in the WMR and genotype 0 predominant at sKED (Atkinson and Bartholomew, 2010a). Introduced RBT mortality and total parasite density were also significantly lower at sKED than in the
WMR. Thus, stocking introduced RBT to supplement recreational fishing may be the most likely explanation for the amplification of parasites in the WMR.

With dam removal and/or modification, the migration of anadromous fish into the upper KR basin is likely to expand the distribution of the parasite and transport lower basin genotypes into the upper basin, altering parasite dynamics. Chinook salmon historically inhabited the WMR and Sprague Rivers, and are the focus of reintroduction efforts (Hamilton et al., 2005). Although this species becomes infected with genotype II, mortality is correlated with genotype I, a parasite genotype not currently present in the upper basin (Atkinson and Bartholomew, 2010a). Hence, the short-term effects of the high parasite densities in the WMR are likely to be negligible for Chinook salmon. However, the returning adult salmon will introduce genotype I into the upper basin. As conditions in the WMR below the Sprague River are already conducive to parasite propagation, this new parasite genotype is likely to become established, affecting juvenile Chinook salmon that migrate through this area of the river. There is also potential for expanding the distribution of *C. shasta* into the Sprague River, as adult Chinook salmon take advantage of spawning habitat. Potential effects from reintroduction of coho salmon are less understood. If these fish migrate into the WMR, the current high densities of parasite genotype II in the WMR are a concern because this genotype has been associated with mortality in this fish species (Atkinson and Bartholomew, 2010a). However, the coho survival after exposure in the upper basin suggests that genotype II may have different sub-genotypes, with one sub-genotype adapted to RBT in the upper basin and one sub-genotype adapted to coho in the lower basin.
ACKNOWLEDGEMENTS

We thank all those who provided assistance in the field; Bill Tinniswood and Roger Smith from the Oregon Department of Fish and Wildlife (ODFW), the Nature Conservancy, private landowners (Lonesome Duck Resort, Waterwheel Campground, Williamson River Resort and Sportsman’s park in Keno), as well as Julie Alexander, Michelle Jordan, Gerri Buckles and Shawn Harris from the Bartholomew laboratory at Oregon State University (OSU). In addition thank you to many members of the Bartholomew Laboratory at Oregon State University who aided in the processing of samples and for their comments and suggestions on this manuscript: Sascha Hallett, Stephen Atkinson, Steve Christy, Matt Stinson, Jill Pridgeon, Adam Ray and Sarah Bjork. Special thanks to Kym Jacobson at the Hatfield Marine Science Center and Kate Field (OSU) for their advice and comments on this manuscript. We would also like to thank the Roaring River Fish Hatchery for fish. Funding was provided by the ODFW Restoration and Enhancement Program.
REFERENCES


Atkinson S.D., Bartholomew, J.L., 2010b Spatial, temporal and host factors structure the Ceratomyxa shasta (Myxozoa) population in the Klamath River basin. Infection, Genetics and Evolution 10, (7), 1019-1026.


Committee (Committee on Threatened and Endangered Fishes in the Klamath River Basin) and NRC (National Research Council). 2004. Endangered and threatened fishes in the Klamath


Udey, L.R., Fryer, J.L., Pilcher, S.K., 1975. Relation of water temperature to ceratomyxosis in rainbow trout (Salmo gairdneri) and coho salmon (Oncorhynchus kisutch). Journal of Fisheries Research Board of Canada 32, 1545-1551.


Figure 2.1: Williamson River and tributaries with the Klamath River basin in the left insert. Large hollow circles indicate river reaches. Water sample sites are depicted with filled circles; triangles represent sentinel fish exposure sites and polychaete (*Manayunkia speciosa*) collection sites are indicated within the hollow boxes.
Figure 2.2: *Ceratomyxa shasta* cycle threshold (Cq) values and temperatures for Williamson River water samples collected in 2008, 2009 and 2010 by reach. Spring Creek and Sprague River confluences of the Williamson River occur at Rkm 27.5 and 19, respectively, as indicated by the vertical lines. Note: two separate sites occur between Rkm 26 and 28, with the lower Cq and temperature values correlating with the site in Reach 2.
Figure 2.3: Quadratic regression plot of temperature versus *Ceratomyxa shasta* cycle threshold (Cq) values for all months and reaches. Main-stem Williamson River samples are indicated using filled squares and tributary data are depicted with hollow squares. The dashed horizontal line indicates parasite production (1 parasite L\(^{-1}\)).
Figure 2.4: *Ceratomyxa shasta* parasite genotype composition from water samples collected in the Williamson River during all four sampling periods. R1=the mouth of the Williamson River to the Sprague River confluence, R2=Sprague River confluence to the Spring Creek confluence and R3=above the Spring Creek confluence to Rkm 42. Each bar represents one liter of water from one sample site within the designated reach.
Figure 2.5: Mortality of non-native rainbow trout (bars) and average *Ceratomyxa shasta* cycle threshold (Cq) values (triangles) for Keno Eddy (sKED) and the Williamson River (sWMR) sentinel sites for years 2006-2010. Letters indicate statistically significant differences of mortality and c<sub>q</sub> between sites. The dashed line designates the c<sub>q</sub> value that corresponds with one parasite L<sup>–1</sup>. 
CHAPTER 3: VIRULENCE OF *CERATOMYXA SHASTA* GENOTYPES I AND II IN ALLOPATRIC AND SYMPATRIC SALMONIDS

Charlene N. Hurst and Jerri L. Bartholomew
ABSTRACT

*Ceratomyxa shasta* is a myxozoan parasite of salmonid fish. In natural communities, distinct genotypes of the parasite are associated with different salmonid hosts. To test the hypothesis that *C. shasta* genotypes demonstrate host-specific virulence, the polychaete host was experimentally infected with myxospores of different genotypes or biotypes. Parasite myxospores of genotype I from Iron Gate (IG) Chinook salmon (*Oncorhynchus tshawytscha*), genotype II from IG coho salmon (biotype IIC, *O. kisutch*) and genotype II from rainbow trout (biotype IIR, *O. mykiss*) were collected from intestines of infected fish. The resulting actinospores were used in three separate trials to challenge four strains of salmonid; low susceptibility strains of Chinook and coho salmon, and highly susceptible strains of Chinook salmon and rainbow trout. Differences in laboratory exposed fish mortality were based on host strain susceptibility to parasite genotype. Genotype I caused mortality in both Chinook strains, although mortality in the highly susceptible Chinook also occurred from exposure to biotype IIR. Biotypes IIR and IIC caused mortality in rainbow trout, whereas IG coho mortality occurred only after exposure to biotype IIC. Thus, genotype I demonstrated host-specific virulence for Chinook salmon, while genotype II was virulent in Chinook and coho salmon and rainbow trout.

Keywords: Myxozoa, virulence, genotype, infection, transmission
INTRODUCTION

The life cycle of the myxosporean parasite, *Ceratomyxa shasta*, requires a polychaete (*Manayunkia speciosa*) and a salmonid host. Polychaete infections occur after contact with myxospores produced in the salmonid and released into the water column. The parasite then proliferates in the polychaete and develops into the waterborne actinospore parasite stage infectious for fish (Bartholomew et al., 1997). Infection with *C. shasta* in fish may result in the disease ceratomyxosis, characterized by severe hemorrhage, inflammation and necrosis of the fish hosts’ intestine, which can be fatal (Bartholomew, 2001).

The degree of the parasite’s pathogenicity, or virulence (Wood and Davis, 1980 see Casadevall and Pirofski, 1999), in salmonids is dependent on factors influencing the host-parasite interaction including host susceptibility to different parasite genotypes. Virulence of each of the parasite genotypes is unknown, but can be assessed by analyzing differences in salmonid mortality or mortality rate. Salmonid susceptibility differs even between strains of the same species: fish strains sympatric (overlapping in range) with the parasite in the basins of the Pacific Northwest of North America are less susceptible than fish strains allopatric (non-overlapping in range) with the parasite (Zinn et al., 1977; Johnson et al., 1979; Ching and Munday, 1984; Bartholomew et al., 1989; Hendrickson et al., 1989; Bartholomew, 1998; Bartholomew et al., 2001). In the Klamath River (KR) basin, Oregon-California (Figure 3.1), four parasite genotypes were characterized by differences in the internal transcribed spacer region I (ITS-1) of the rRNA operon (Atkinson and Bartholomew, 2010a, b). Among the native salmonids in the KR, these genotypes were associated with different hosts: genotype 0 was isolated from steelhead (*Oncorhynchus mykiss*, anadromous form) and redband trout (*O. mykiss newberrii*), genotype I from Chinook salmon (*O. tshawytscha*) and genotype II from coho salmon (*O. kisutch*). Genotype II also infected introduced rainbow trout (*O. mykiss*, freshwater form). Another genetic variant, genotype III, was found in minor proportions in a variety of hosts.
The division of the KR basin into an upper and lower basin by a series of hydroelectric dams prevents anadromous fish passage into the upper basin. Since the distribution of parasite genotypes in the basin correlated with the presence of their associated salmonid host, differences in disease dynamic, between the upper and lower basin have been observed (Foott et al., 2004; Stocking et al., 2006; Atkinson and Bartholomew, 2010a). In the upper Klamath basin, exposures of native sympatric strains of fall Chinook and coho salmon resulted in little to no infection from \textit{C. shasta} genotypes present above the dam (0, II, III), but mortality occurs in both species in the lower Klamath basin (Atkinson and Bartholomew, 2010a). The extirpation of Chinook salmon from the upper basin may have limited the distribution of genotype I to the lower Klamath basin (Atkinson and Bartholomew, 2010a). However, genotype II is present at high densities in some areas of the upper basin likely from the stocking of rainbow trout (Atkinson and Bartholomew 2010a), and thus survival of coho salmon was unexpected. This led to the hypothesis that genotype II may exist as two biotypes: IIC supported by sympatric coho salmon and IIR by introduced, allopatric rainbow trout.

To test the hypothesis of host-specific virulence of \textit{C. shasta} genotypes we conducted laboratory cross-infection experiments exposing several strains and species of salmonids to parasite genotypes I and II. Our study focused on these genotypes because they are associated with mortality in fall Chinook (I) and threatened coho salmon (II) listed under the Endangered Species Act. Our first objective, addressed in trials 1 and 2, was to determine if genotype virulence differed between allopatric and sympatric salmonid strains. We expected equal mortality levels from all genotypes in highly susceptible fish and differential mortality in less susceptible fish. To test the hypothesis that genotype II exists as different biotypes, trial 3 assessed the virulence of IIC and IIR in coho salmon and susceptible rainbow trout. Understanding the role of each parasite genotype in the pathogenicity of the fish host is important for predicting how disease dynamics may change when the hydroelectric dams are removed and anadromous salmonids repopulate their historic ranges.
MATERIALS AND METHODS

Polychaete colony establishment: *Manayunkia speciosa* was collected from Klamath Lake (42°27.823N, 121°57.288W) in September 2008 for trial 1 and 2 and in May 2010 for trial 3 to establish a laboratory population. This locality was chosen because of the high densities of polychaetes and low prevalence of *C. shasta* infection (0.45%) in the population (Stocking and Bartholomew, 2007). Polychaetes and associated sediment were collected according to Bjork and Bartholomew (2009) and then transported to the John L. Fryer Salmon Disease Laboratory (SDL) at Oregon State University, Corvallis, OR, USA. Equal portions of sediment were placed into three pairs of 60L (15.5 gallon) plastic tubs and allowed to settle for 24 hours. These tubs were supplied with a constant flow of water (~1L/min) from the Willamette River, which was UV-treated to ensure that viable *C. shasta* from the Willamette system were not introduced.

Quantification of polychaetes: A plastic grid divided into 170 cells (27cm³) was placed over the surface of the sediment in each tub. Ten cells were randomly selected from the grid; 10mL of sediment was collected from each cell using a plastic pipette and placed into a Petri dish. *M. speciosa* were separated using modified dental picks and identified using a dissecting microscope at 10x power. The number of *M. speciosa* in each cell was used to estimate polychaete densities at the beginning and end of the trial(s).

Myxospore source: As a source of genotype I myxosposes, sympatric Chinook salmon from Iron Gate (IG) Hatchery (Hornbrook, CA), were exposed above Beaver Creek, CA, (41°52.037N, 122°48.582W). Additional genotype I myxosposes were harvested from adult Chinook salmon carcasses collected from Bogus Creek, CA (41°55.753N, 122°26.602W). As a source of genotype IIR, allopatric rainbow trout from Roaring River Hatchery (Scio, OR) was exposed in the Williamson River (42°30.820N, 121°55.021W). As a source of genotype IIC, sympatric coho salmon from IG Hatchery were exposed above Beaver Creek, CA (Figure 3.1). All exposed fish were held at the exposure site for 72 hours and then monitored at the SDL as previously described.
(Stocking et al., 2006). From each fish, a piece of intestine was frozen for sequencing of the parasite to confirm parasite genotype identity (see below for molecular methodology).

Establishing infected colonies of each genotype: Myxospores were isolated from fish intestines according to Bartholomew et al. (1989b), except cheesecloth lined a plastic funnel in place of the Tyler® sieves for the removal of large pieces of tissue.

Approximately 50µL of a 1mL parasite suspension was added to the hemocytometer and myxospores were counted in all four sections of the grid. Averaged counts from the four sections were multiplied by 1 x 10⁴ to determine parasites mL⁻¹. Approximate numbers of C. shasta spores used to inoculate the colonies were 5 x 10⁶ of genotype I, and 3 x 10⁸ of biotype IIR for trials 1 and 2 and 1 x 10⁶ of IIR and 2.2 x 10⁶ of IIC for trial 3. Myxospore dose varied due to availability of the appropriate fish host. Spores were immediately mixed with 1L of UV-treated river water and used to inoculate one pair of polychaete tubs with either genotype I (genotype I treatment) or genotype II (genotype II treatment) in trials 1 and 2 and with either genotype IIR (IIR treatment) or IIC (IIC treatment) in trial 3. One pair of polychaete tubs was not inoculated to assess the baseline level of infection in polychaetes (polychaete control).

Cross-infection experimental design: The outflow from the polychaete tubs was directed into three glass 378 L (100 gallon) aquaria placed on a shelf below the plastic tubs. An additional aquarium was supplied directly with UV treated river water to control for parasites that may have survived UV treatment (water control). Each aquarium contained one Odyssey WP500 water pump to re-circulate water from the aquarium to the two corresponding polychaete tubs that supplied it (Figure 3.2). Each aquarium was partitioned into four using plastic dividers and 0.3cm extruded plastic netting (Internet Inc.). This facilitated water flow through all sections of the aquarium and permitted simultaneous challenge of different fish groups. Temperatures in two aquaria were recorded using automated loggers (HOBOware, Onset, Bourne, MA).

Parasite dose monitoring: One L of water was collected from each fish tank bi-weekly from December 4, 2008 to July 2, 2009 in trials 1 and 2. In trial 3, frequency of water sampling was increased to twice a week beginning September 1, 2010 and ending
November 1, 2010. All water and control samples were processed for assay by quantitative PCR (qPCR) according to Hallett and Bartholomew (2006). Tests for water sample inhibition were conducted on one water sample from each fish tank. A difference of greater than one cycle indicated the occurrence of inhibition and samples were then re-analyzed with a 1:10 dilution using molecular grade water. The quantitative cycle threshold (Cq) value of a one spore reference sample was used to determine parasites L⁻¹. A Difference of 3.3 cycles was used to distinguish between each 10 fold increase in parasite density (Hallett and Bartholomew, 2006). Exposure doses were estimated using Cq values and water velocity measured from the aquaria inflow.

**Fish cross-infection challenges:** Allopatric rainbow trout (RBT) were from either Roaring River (Scio, OR) or Trout Lodge (Hood River, OR) fish hatcheries. Sympatric strains of fall Chinook (CHF) and coho salmon (coho) were from Iron Gate (IG) Hatchery (Hornbrook, CA). An allopatric Salmon River fall Chinook salmon (SR CHF) strain was from Salmon River Hatchery (Otis, OR). Fish were added when parasite abundance in the inoculated aquaria was >10 parasites L⁻¹. Initiation dates, fish numbers and sizes varied with each trial depending on fish availability and are summarized in Table 3.1. In trials 1 and 2, fish were exposed for 120 days. In trial 3, fish were held in the inoculated aquaria for 30 days and then moved into specific-pathogen free fiberglass tanks for an additional 30 days. Fish were fed a commercial diet daily (Bio-Oregon, Longview, WA) and monitored for signs of disease. All fish were euthanized using tricaine methanosulfate (MS-222, Argent Laboratories, Redmond, WA) and a sample of the intestine (~75mg) was collected and frozen for determination of genotype.

**Parasite transmission:** To ensure parasite transmission occurred from the IIR and IIC exposures in trial 3, 5 IG coho salmon from each exposure aquaria and 3 from each of the control (polychaete and water) aquaria were euthanized at 10 days post-exposure. Intestines were collected and assayed using a *C. shasta*-specific PCR (Palenzuela *et al.*, 1999).

**Determination of parasite genotype:** DNA from all fish intestines and qPCR positive water samples (>1 parasite L⁻¹) were assayed in two rounds of PCR in 20µL
reactions using the forward primer Cs1482F and the reverse primer CsgenR1 (Atkinson and Bartholomew, 2010a, b). PCR products were purified using Exosapit (USB, Cleveland, OH) and products were sequenced according to the methods in Atkinson and Bartholomew (2010a). Sequences were aligned using Bioedit (Hall, 1999) and genotypes were determined visually, with proportions of each genotype in mixed genotype samples quantified using peak height ratios (Atkinson and Bartholomew, 2010a).

Statistical analyses: Degree-days to morbidity were used to account for temperature differences between trials and were calculated by summing the average daily temperature over the course of the experiment. Exposure doses (log10 transformed for normalization) and degree-days to morbidity for allopatric CHF in trial 2 and allopatric RBT in trial 3 were compared using a one-way ANOVA. Significant differences within ANOVA’s were determined using the Tukey test. All statistical analyses were performed using S-plus version 8.1.1 statistical software (TIBCO, Palo Alto, CA). Groups were considered significantly different if the p-value <0.05.

RESULTS

Quantification of polychaetes: Polychaete densities numbering in the thousands were sufficient for laboratory cross-infection experiments. In trials 1 and 2, the number of polychaetes in the polychaete control and genotype I or II treatments were estimated at 6.8 x 10^3, 8.0 x 10^3, and 5 x 10^3 polychaetes respectively. In trial 3, polychaete densities were approximately 2.4 x 10^3 in the polychaete control, 1.0 x 10^3 in the IIC treatment and 1.6 x 10^3 in the IIR treatment. Polychaete densities were assessed again at the end of the experiments to estimate percent survival. No polychaetes survived in the treatments from trials 1 and 2, and the population was reduced by 85% in the polychaete control. In trial 3, no polychaetes survived in the polychaete control or IIC treatment groups and the population was reduced by 86% in the IIR treatment group.

Parasite dose monitoring: Exposure doses varied between exposure aquaria during all trials. Parasite dose was not significantly different between exposures in trials 1 and 2 (one-way ANOVA, trial 1: F(2, 27) = 1.11, p = 0.34; trial 2: F(2, 18) = 3.39, p = 0.056). Parasite dose in exposures ranged from 5 to 6.0 x 10^6 parasites day^{-1} in trial 1 and
348 to $2.5 \times 10^5$ parasites day$^{-1}$ in trial 2. Exposure doses in trial 3 were only significantly different between the polychaete control and the biotype IIC treatment (one-way ANOVA, $F(2, 27) = 4.47, p = 0.021$, Tukey 2.48) with parasites ranging from 39 to $7.2 \times 10^3$ in the polychaete control, $3.0 \times 10^4$ to $1.6 \times 10^6$ in the IIR treatment and $4.0 \times 10^4$ to $1.6 \times 10^7$ in the IIC treatment. Variability in samples was not due to sample processing, as an inter-assay variability of 0.7 cycles was calculated for all qPCR assays performed.

Parasite genotypes in water samples varied over the course of the experiment. During trials 1 and 2, genotype II predominated in the polychaete control, although minor amounts of genotype III and 0 were detected in February and May. In trial 3, parasite densities in the polychaete control aquarium were $< 1$ parasite L$^{-1}$, below the sequencing threshold. In all genotype II treatments, only genotype II was identified in water samples. A combination of genotypes I and II were present throughout the genotype I treatment exposure (as a result of the background infection in the polychaetes). In trial 1, 60% of the parasites were genotype I until mid-February; thereafter, only genotype II was detected. In trial 2, parasite ratio was 50:50 genotypes I and II, with a minor proportion of genotype 0 parasites detected in late May. Genotype proportions for each sampling time point are included in Table 3.2.

Fish cross-infection challenges: *Ceratomyxa shasta* infections resulted in mortality in all species/strains of fish. In trial 1, which examined the effect of genotype on pathogenicity in a sympatric and allopatric host species, 70% mortality from genotype II occurred in allopatric RBT exposed in the polychaete control. In both the genotype I and II treatments, 100% of the RBT died with genotype II infections. There was no mortality in sympatric IG CHF exposed in either the polychaete control or genotype II treatment, although infections with genotype II occurred in 50% of the fish in the polychaete control and 100% of the fish in the genotype II treatment. Infections of IG CHF from genotype I in the genotype I treatment resulted in 87.5% mortality (Figure 3.3a).

Trial 2 expanded on trial 1 and incorporated additional sympatric and allopatric fish strains. In this trial, RBT succumbed to infections in all exposures from genotype II,
with 20% mortality in the polychaete control and 100% mortality in both genotype I and genotype II treatments. Mortality of the allopatric SR CHF was similar to RBT, with 70% mortality in the polychaete control, and 100% in each of the treatments (Figure 3.3b). In the genotype I treatment, mortality in SR CHF was caused by genotype I, whereas RBT died from genotype II. Degree-days to morbidity were not significantly different between SR CHF infected with genotype I in the genotype I treatment and those infected with genotype II in the genotype II treatment (Figure , one-way ANOVA, F (2, 23) =181.3 , p < 0.001, Tukey 2.55). One IG coho died from infection with genotype I, but no infections resulted from exposures to genotype II.

The objective of trial 3 was to determine if genotype II from RBT and genotype II from coho salmon had differing levels of virulence in different fish host species. Mortality of coho salmon was a result of infection by genotype II in both treatments, with 10% in the IIR treatment and 100% in the IIC treatment; no mortality occurred in the polychaete control. Similar to previous trials, RBT mortality was 100% in all exposures (Figure 3.3c). However, the mortality rate of RBT exposed to IIR was significantly faster than in the IIC treatment (Figure 3.5, one-way ANOVA, F (2, 27) = 15.77, p < 0.001, Tukey 2.48). Mortalities of RBT from the third trial were due solely to genotype II, except in the polychaete control where 40% of the RBT died of mixed genotype II and III infections. C. shasta was not detected in any fish held in UV treated water from the Willamette River during any trial.

Parasite transmission: Sampling of RBT and IG coho at 10 days post-exposure in trial 3 demonstrated that parasites in the IIR and IIC treatments were transmitted to both fish species. In the IIR treatment 2/5 IG coho were infected, compared with 5/5 fish infected in the IIC treatment and 2/3 coho infected in the polychaete control. All RBT were infected in both treatments and in the polychaete control. At the end of the experiment, surviving IG coho in the polychaete and water control were uninfected, but 2/8 IG coho were infected in the genotype IIR treatment.

DISCUSSION
This study provides empirical support for the hypothesis presented in Atkinson and Bartholomew (2010a) that particular *C. shasta* genotypes cause mortality in certain salmonid hosts. In laboratory cross-infection experiments, we demonstrated host-specific virulence for genotype I, as mortality only occurred in Chinook salmon. However, mortality in two different species, RBT and Chinook salmon (trial 2), suggested virulence of genotype II is not host-specific. Differential mortality of coho salmon to genotype II from two different host species in separate geographical regions (trial 3) suggested that biotypes of genotype II exist. Therefore genotype I appeared to be a specialist, causing mortality in only one host species, while genotype II was a generalist lethal for multiple salmonid hosts.

Laboratory cross-infection challenges of sympatric fish strains require large numbers of parasites. To obtain the necessary doses, we used natural polychaete populations from Klamath Lake, OR, where parasite genotypes 0, II and III occur (Atkinson and Bartholomew, 2010a). A subset of polychaetes was monitored for parasites resulting from natural infections, and indeed, fish kept in the polychaete control aquarium developed ceratomyxosis. Water sample data from the polychaete control demonstrated the presence of genotype II and minor amounts of genotype 0 and III; the same genotypes identified previously from Klamath Lake. No fish became infected in the water control, indicating that Willamette River water was an unlikely source of parasites. Although the use of natural populations of polychaetes did result in mixed genotypes in these challenges, this did not inhibit our ability to determine the virulence of parasite genotypes in different fish strains.

Simultaneous exposures of sympatric (IG CHF) and allopatric (SR CHF) Chinook salmon to each parasite genotype demonstrated how virulence differs with fish strain. SR CHF is a coastal strain reared in waters where the parasite is absent; thus the development of resistance was unnecessary (Zinn *et al.*, 1977; Bartholomew, 1998; Bartholomew *et al.*, 2001). In contrast, the IG CHF strain is relatively resistant to ceratomyxosis (Ray *et al.*, 2010; Bjork, 2010). In sympatric CHF genotype I caused mortality, while exposure to biotype IIR did not result in disease. The survival of IG CHF
following exposure to genotype IIR (from RBT) suggests sympatric CHF have some resistance mechanism(s) that allow for their survival after infection with this biotype. In contrast, mortality of allopatric CHF occurred after exposure to genotype I and biotype IIR. Although mean day to morbidity was not significantly different between exposures, mortality only occurred from biotype IIR in the absence of genotype I. Thus, genotype I was more virulent in both strains of Chinook salmon than biotype IIR. In addition, genotypes of low virulence in sympatric fish strains were more virulent in allopatric fish strains.

Currently, the only distinguishing criterion between C. shasta genotypes is a trinucleotide repeat in the internal transcribed spacer 1 region of the rRNA operon (Atkinson and Bartholomew, 2010a). However, further separation of genotype II into biotypes IIR and IIC based on host species (RBT and coho salmon, respectively), supports both laboratory findings and field observations. In field studies, mortality of coho salmon exposed in the lower Klamath River was a result of infection by genotype II. However, coho salmon did not become infected during exposure in the upper basin despite high densities of the same parasite genotype (Atkinson and Bartholomew, 2010a). Similarly, in laboratory trials coho salmon survived exposure to parasite biotype IIR (trials 2 and 3) but succumbed to infection by biotype IIC. The allopatric RBT died after exposure to both biotypes, although biotype IIR was more virulent. Thus, infection of multiple host species suggests genotype II has evolved two biotypes, one of which has adapted to a new host; stocked rainbow trout in the upper basin.

The high virulence of a parasite genotype in a specific host population may correlate with low virulence of this same parasite genotype in other hosts (Woolhouse et al., 2001 in Rigaud et al., 2010; Mackinnon et al., 2002). Our results lend support to the hypothesis that certain genotypes are adapted for particular hosts. Biotype IIR was transmitted to sympatric CHF and coho but infection did not result in mortality, yet IIR caused high mortalities in allopatric RBT. Genotype I was lethal for CHF and was not detected in RBT or coho. Biotype IIC caused mortality in coho, but was less virulent in RBT than biotype IIR. These data suggest that high virulence of C. shasta genotype I in
sympatric CHF, biotype IIR in allopatric RBT and biotype IIC in sympatric coho may be associated with their lower virulence in other salmonids.

Parasite genotype interactions within the host, in combination with the host immune response, may dictate the outcome of infections. Infection with biotype IIR in sympatric CHF and coho did not result in disease, and mortality from genotype II only occurred in allopatric CHF when genotype I was absent. Theoretical models demonstrated that when multiple genotypes of a parasite are transmitted, competition for resources may result in increased genotype virulence (Mosquera and Adler, 1998). In addition, the fish host’s immune system may be capable of containing or eliminating certain parasite genotypes before proliferation in the intestine occurs (Bjork, 2010). In this study, when allopatric CHF were exposed simultaneously to genotypes I and biotype IIR, genotype I was the only genotype detected, suggesting genotype I successfully outcompeted biotype IIR. In contrast, the detection of biotype IIR in sympatric CHF suggests that biotype IIR is contained or eliminated while genotype I proliferates and causes disease. Infection, but no disease with certain genotypes support a virulence trade-off in different hosts (Rigaud et al., 2010), potentially through either parasite competition or host resistance mechanisms.

The virulence of different parasite genotypes may also differ depending on the life history of the fish. The virulence of genotype I and biotype IIC may be well-suited for a semelparous fish host. If returning salmon become infected after re-entry, the parasite has a limited amount of time before the fish host spawns and dies. In this case, high virulence would be more favorable if correlated with a higher parasite reproductive output within the short amount of time available to the host. In contrast, steelhead and redband trout infections with genotype 0 (Atkinson and Bartholomew, 2010b) highlight the opposite trend. In iteroparous hosts, a parasite with low virulence may be more beneficial, leading to low levels of parasite reproduction over a longer time frame, potentially resulting in greater parasite output overall.

With the proposed removal of four of the five dams on the main-stem Klamath River, management decisions regarding which fish strains should be reintroduced into the
upper basin should take into consideration the susceptibility of each fish strain to all genotypes/ biotypes of *C. shasta*, for three reasons. First, reintroduced fish strains that do not have inherent resistance to the parasite are likely to die from a variety of parasite genotypes, as demonstrated in allopatric CHF exposures. Second, spring Chinook salmon rear in lower Klamath River tributaries (Hooten and Smith, 2008) where the parasite is not detected, and enter the system below the area of high parasite density (Stocking *et al.*, 2006). Therefore, spring Chinook may be more susceptible to genotypes/biotypes of the parasite that are less virulent in fall Chinook salmon, such as biotype IIR. Third, the rate of virulence evolution in these genotypes is unknown and higher virulence may evolve when parasite genotypes are exposed to new hosts (i.e. IIR to coho salmon). Thus, anadromous fish strains used for reintroduction into the upper Klamath basin should be those with relatively low susceptibility to all parasite genotypes to ensure a successful reintroduction.
ACKNOWLEDGEMENTS

We acknowledge Iron Gate, Trout Lodge, Salmon River and Roaring River hatcheries for contributing fish, Stephen Atkinson for assistance with parasite genotyping, Don Stevens and Shawn Harris for experimental set-up, Harriet Lorz and Jill Pridgeon for assistance with polychaete counting and culture, Scott Foott and Rich Holt for providing fish for myxospores, Gerri Buckles, Sascha Hallett and Matt Stinson for assistance with PCR and qPCR, Sarah Bjork, Julie Alexander and Adam Ray for their manuscript advice. We also thank the Oregon Department of Fish and Wildlife Restoration and Enhancement Program for funding.
REFERENCES


Bjork, S.J., Bartholomew, J.L., 2010. Invasion of *Ceratomyxa shasta* (Myxozoa) and comparison of migration to the intestine between susceptible and resistant fish hosts. International Journal for Parasitology 40, 1087-1095.

Bjork, S.J., 2010. Factors affecting the *Ceratomyxa shasta* infectious cycle and transmission between polychaete and salmonid hosts. Doctoral Dissertation, Oregon State University, Corvallis, OR.

Casavellas, A., Pirofski, L., 1999. Host-pathogen interactions: redefining the basic
concepts of virulence and pathogenicity. Infection and Immunity 67, (8), 3703-3713.


*Ceratomyxa shasta*. Diseases of Aquatic Organisms 93, 63-70.


Figure 3.1: Map of the Klamath River basin. The upper and lower portions of the basin are designated with shaded circles. Key to sites: star = polychaete collection, triangle = Williamson River, circle = Beaver Creek, square = Bogus Creek.
Figure 3.2: Cross-infection study experimental set-up. Paired polychaete tubs are on the top rack, fish exposure aquaria are on the bottom rack. Each pair of polychaete tubs supplies parasites for one fish aquaria. The UV sterilization apparatus is at left.
Table 3.1: Fish cross-infection challenge experimental design: IG=Iron Gate, SR=Salmon River, RBT=rainbow trout, CHF= Chinook salmon. Three aquaria were used for each trial: 1) polychaete control, 2) genotype I treatment, 3) genotype II treatment (trials 1 and 2) and 1) polychaete control, 2) biotype IIR 3) biotype IIC (trial 3).

<table>
<thead>
<tr>
<th>Trial/Date Initiated</th>
<th>Fish Species</th>
<th>Fish Strain, Susceptibility</th>
<th># / Aquaria</th>
<th>Size (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a December 12, 2008</td>
<td>RBT</td>
<td>Cape Cod 72, high</td>
<td>5</td>
<td>30-40</td>
</tr>
<tr>
<td></td>
<td>CHF</td>
<td>IG, low</td>
<td>5</td>
<td>20-30</td>
</tr>
<tr>
<td>1b February 12, 2009</td>
<td>RBT</td>
<td>Cape etc cod 72, high</td>
<td>5</td>
<td>30-40</td>
</tr>
<tr>
<td></td>
<td>CHF</td>
<td>IG, low</td>
<td>3</td>
<td>20-30</td>
</tr>
<tr>
<td>2 April 18, 2009</td>
<td>RBT</td>
<td>Cape cod 72, high</td>
<td>5</td>
<td>5-10</td>
</tr>
<tr>
<td></td>
<td>CHF</td>
<td>IG, low</td>
<td>20</td>
<td>5-10</td>
</tr>
<tr>
<td></td>
<td>CHF</td>
<td>Salmon River, high</td>
<td>10</td>
<td>5-10</td>
</tr>
<tr>
<td></td>
<td>coho</td>
<td>IG, low</td>
<td>20</td>
<td>5-10</td>
</tr>
<tr>
<td>3 September 1, 2010</td>
<td>RBT</td>
<td>Cape cod 72, high</td>
<td>15</td>
<td>30-60</td>
</tr>
<tr>
<td></td>
<td>coho</td>
<td>IG, low</td>
<td>13 controls</td>
<td>30-60</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>15 treatments</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3.3 (a-c): Laboratory cross-infection challenges with genotypes/biotypes of *Ceratomyxa shasta*. Trial 1 exposure results of (a) allopatric rainbow trout (RBT) and sympatric Chinook (CHF). Trial 2 results (b) depicting exposures of allopatric RBT and allopatric CHF. Trial 3 results (c) showing exposures of sympatric coho and allopatric RBT. Each shape indicates an individual fish. Fish survivors positive for the parasite are on the right side of the dashed vertical black line. The y-axis indicates which genotype was detected in the intestinal tissue of positive fish. Fish survivors in which C. *shasta* was not detected are not shown.
Figure 3.4 Mean degree day to morbidity of allopatric Salmon River Chinook exposed in trial 3. Letters indicate those exposures that are statistically different from each other (one-way ANOVA, \( p < 0.001 \)).
Figure 3.5 Mean degree day to morbidity of allopatric rainbow trout exposed in trial 3. Letters indicate those exposures that are statistically different from each other (one-way ANOVA, $p < 0.001$).
Table 3.2: *Ceratomyxa shasta* parasite genotype identity and genotype proportion in water collected from the polychaete control and treatment aquaria during the first two trials. Water was collected biweekly from the first two trials. BST = below sequencing threshold and designated those samples for which there was not enough parasite DNA (< 1 parasite L$^{-1}$) to determine parasite genotype.

<table>
<thead>
<tr>
<th>Trial</th>
<th>Month/Year</th>
<th>Temp</th>
<th>Genotype</th>
<th>Proportion</th>
<th>Genotype</th>
<th>Proportion</th>
<th>Genotype</th>
<th>Proportion</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Dec-08</td>
<td>8.1</td>
<td>BST</td>
<td>BST</td>
<td>I</td>
<td>100</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>5.4</td>
<td>BST</td>
<td>BST</td>
<td>BST</td>
<td>I/II</td>
<td>76/24</td>
<td>BST</td>
<td>BST</td>
</tr>
<tr>
<td></td>
<td>Jan-09</td>
<td>8.7</td>
<td>BST</td>
<td>BST</td>
<td>BST</td>
<td>BST</td>
<td>BST</td>
<td>BST</td>
</tr>
<tr>
<td></td>
<td>Feb-09</td>
<td>12.9</td>
<td>BST</td>
<td>BST</td>
<td>II</td>
<td>100</td>
<td>BST</td>
<td>BST</td>
</tr>
<tr>
<td></td>
<td>10.7</td>
<td>II/III/0</td>
<td>60/30/10</td>
<td>I/II</td>
<td>68/32</td>
<td>II</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>9.4</td>
<td>BST</td>
<td>BST</td>
<td>BST</td>
<td>I</td>
<td>100</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Mar-09</td>
<td>13.7</td>
<td>II</td>
<td>100</td>
<td>II</td>
<td>100</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>12.3</td>
<td>II</td>
<td>100</td>
<td>II</td>
<td>100</td>
<td>II</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Apr-09</td>
<td>14.8</td>
<td>II</td>
<td>100</td>
<td>II</td>
<td>100</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>Apr-09</td>
<td>16.3</td>
<td>II</td>
<td>100</td>
<td>I/II</td>
<td>75/25</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>May-09</td>
<td>14.4</td>
<td>II/0</td>
<td>89/11</td>
<td>I/II</td>
<td>53/37</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>16</td>
<td>II</td>
<td>100</td>
<td>II/0</td>
<td>85/15</td>
<td>II</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jun-09</td>
<td>18.6</td>
<td>II</td>
<td>100</td>
<td>I/II</td>
<td>35/65</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>17.6</td>
<td>II</td>
<td>100</td>
<td>I/II</td>
<td>54/46</td>
<td>II</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Jul-09</td>
<td>19.7</td>
<td>II</td>
<td>100</td>
<td>I/II</td>
<td>66/34</td>
<td>II</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>II</td>
<td>100</td>
<td>I/II</td>
<td>85/15</td>
<td>II</td>
<td>100</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 4: SUMMARY
SUMMARY

Obtaining information about the density, distribution and genotype composition of the myxozoan parasite, *Ceratomyxa shasta*, in the Williamson River (WMR) and its two tributaries was necessary for determining potential effects of the parasite on reintroduced anadromous salmonids into the upper basin. Previous studies have identified the presence of the parasite until the Sprague River confluence at River kilometer (Rkm) 19 using sentinel fish (Hemmingsen *et al.*, 1988; Buchannan *et al.*, 1989). However, the parasite distribution above the Sprague River confluence, the density of parasites above upper Klamath Lake and parasite genotype composition in the WMR had never been documented. Based on parasite density and distribution results as well as geographical features, the main-stem WMR was divided into three reaches.

High parasite densities occurred from the mouth of the WMR to the Sprague River confluence (Reach 1) likely due to host overlap and appropriate environmental conditions. Between the Sprague River and Spring Creek tributaries (Reach 2), parasite was not detected, likely due to cooler temperatures which restrict fish infection and actinospore release (Schafer, 1968; Johnson *et al.*, 1975; Udey *et al.*, 1975; Ratliff, 1983; Hendrickson *et al.*, 1989; Bjork, 2010). In the area above Spring Creek (Reach 3), parasite densities between the Spring Creek confluence to river kilometer (Rkm) 33 were within the range of 1-10 parasites L⁻¹, but parasite was not detected above Rkm 33. The identification of low polychaete densities in this reach as compared to below the Sprague River suggested that the environment was not stable enough to allow for large polychaete densities. *C. shasta* was undetected in the Spring Creek and Sprague River tributaries, likely as a result of temperature constraints. Parasite genotype composition was dominated by genotype II isolated from non-native rainbow trout (RBT, *Oncorhynchus mykiss*), although a minor amount (<30%) of genotype 0 was identified, likely supported by the native redband trout (*O. mykiss newberrii*). Comparison between sites with (WMR) and without (Keno Eddy) fish stocking demonstrated that stocking results in amplification of parasite densities and alters parasite genotype.
Identification of differences in host mortality associated with different \( C. \ shasta \) genotypes has caused us to re-examine our assumptions about how the high parasite densities present in the WMR will affect reintroduction plans. To determine what parasite genotypes would be most likely to cause disease in reintroduced salmonids we tested the virulence of parasite genotypes I and II in a number of allopatric and sympatric fish strains. These genotypes were selected due to the high mortality in ESA-listed threatened Iron Gate (IG) coho (genotype II) and IG Chinook salmon (genotype I) and their predominance in the lower Klamath basin (Atkinson and Bartholomew, 2010a). Cross-infection studies conducted in the laboratory demonstrated that challenge with genotype I resulted in mortality in both sympatric and allopatric Chinook salmon strains (\( O. \ )

\textit{tshawytscha}), but when the same fish strains were challenged with genotype II, mortality only occurred in the allopatric strain. We have also shown that two biotypes of genotype II exist; IIR caused mortality in allopatric RBT and IIC caused mortality in IG coho (\( O. \ )

\textit{kisutch}). Results of these challenge studies indicate that the reintroduction of Chinook and coho salmon into the upper basin may not be adversely affected by the genotypes/biotypes of \( C. \ shasta \) currently present (0 and IIR). However, it is likely that genotype I and biotype IIC will become establish in the upper Klamath basin.

**MANAGEMENT CONSIDERATIONS**

- With the removal of the dams on the Klamath River, anadromous fish utilizing upper basin habitat will likely carry lower basin \( C. \ shasta \) genotypes (i.e. genotype I, IIC) into the upper basin. Cross-infection studies with polychaetes collected from upper Klamath Lake have shown that these polychaete populations are capable of becoming infected with, and producing actinospores of, lower basin parasite genotypes. In addition, current high parasite densities resulting from the stocking of allopatric RBT indicate the potential for propagation of parasite genotypes from the lower basin. Thus introduced parasite genotypes will likely become established in the upper basin.

- \( Ceratomyxa \ shasta \) was not detected from water samples collected in the lower portion of the Sprague River, indicating that the parasite life cycle was not
established in that tributary. This may be due to avoidance by RBT of this tributary in the summer months when temperatures are warmer than in the WMR. In addition, stocked RBT have likely succumbed to infection before temperatures in the Sprague River decrease, suggesting a lack of host overlap in the Sprague. However, spring Chinook infected with *C. shasta* returning in the spring when temperatures are cooler may utilize Sprague River habitat, resulting in the potential establishment of the parasite in this tributary and expansion of the parasite’s range.

- With the reintroduction of steelhead trout, which become infected with genotype 0, the levels of genotype 0 could increase in the upper basin. Thus far, only infection has been documented in native redband trout, but increasing genotype 0 levels could result in native redband trout mortality.
- If strains of salmon from other basins, even basins where *C. shasta* is present, are used for reintroduction, then evaluations of their susceptibility to all parasite strains should be conducted to guarantee that amplification of genotypes problematic for native species does not occur (i.e. IIC for coho) and ensure that out-of-basin fish strains are not susceptible to the parasite genotypes in the Klamath River.

**FUTURE RESEARCH**

- We were not able to test the virulence of *C. shasta* genotypes 0 and III due to their low prevalence in both water and fish in the Klamath River. Understanding of virulence levels of these genotypes in different fish strains is necessary in the event that genotype composition shifts in the future to predominantly 0 and III.
- An assessment of parasite genotype virulence in spring Chinook has not been conducted. Runs of both species in the Klamath River are small and obtaining samples is difficult, but movement of these fish into the upper basin after dam removal could increase mortality in these fish strains if they are susceptible to any of the parasite genotypes from either the lower or upper basin.
• Our studies identified two biotypes of genotype II, but due to limited genetic information on myxozoan parasites we were unable to distinguish genetic differences between these biotypes. This information is needed to identify the biotypes in fish and water samples collected from the field.

• Establishing virulence levels of each parasite genotype in different strains of fish is necessary to identify which genotypes may be of concern for certain fish strains. However, exposure to a single genotype in the river is unlikely; therefore, monitoring the course of parasite interactions within the host after multiple genotype exposures would better model the outcome of natural exposures.

• If prior exposures to less virulent parasite genotypes inhibit infections with more virulent genotypes, then protection against future *C. shasta* infections could occur. The inability of more virulent genotypes to infect could result in reduced fish mortality and/or a reduction in myxospore output, leading to a decreased chance of infection in polychaetes and fewer fish infections the following year.

• Currently, detection of *C. shasta* genotypes within the fish host is not possible, as probes for each genotype have not been developed for use with in situ hybridization techniques. In order to determine if parasite genotype interactions and/or tissue tropism occurs, development of probes for each of the genotypes is essential.
BIBLIOGRAPHY


Bjork, S.J., Bartholomew, J.L., 2010. Invasion of Ceratomyxa shasta (Myxozoa) and comparison of migration to the intestine between susceptible and resistant fish hosts. International Journal for Parasitology 40, 1087-1095.


from the Klamath River. California Fish and Game 90, 71-84.


