AN ABSTRACT OF THE THESIS OF

Sean Robert Roon for the degree of Master of Science in Microbiology presented on December 9, 2014.

Title: Distribution and Coinfection of Microparasites and Macroparasites in Juvenile Salmonids in Three Upper Willamette River Tributaries.

Abstract approved:

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Jerri L. Bartholomew

Wild fish populations are typically infected with a variety of micro- and macroparasites that may affect fitness and survival, however, there is little published information on parasite distribution in wild juvenile salmonids in three upper tributaries of the Willamette River, OR. The objectives of this survey were to document (1) the distribution of select microparasites in wild salmonids and (2) the prevalence, geographical distribution, and community composition of metazoan parasites infecting these fish. From 2011-2013, I surveyed 279 Chinook salmon *Oncorhynchus tshawytscha* and 149 rainbow trout *O. mykiss* for one viral (IHNV) and four bacterial (*Aeromonas salmonicida*, *Flavobacterium columnare*, *Flavobacterium psychrophilum*, and *Renibacterium salmoninarum*) microparasites known to cause mortality of fish in Willamette River hatcheries. The only microparasite detected was *Renibacterium salmoninarum*, causative agent of bacterial kidney disease, which was detected at all three sites. I identified 23 metazoan parasite taxa in these fish. Nonmetric multidimensional scaling of metazoan parasite communities reflected a nested structure with trematode metacercariae being the basal parasite taxa at all three sites. The freshwater trematode *Nanophyetus salmincola* was the most common macroparasite observed at three sites. Metacercariae of *N. salmincola* have been shown to impair immune function and disease resistance in saltwater. To investigate if *N. salmincola* affects disease susceptibility in freshwater, I

conducted a series of disease challenges to evaluate whether encysted *N. salmincola* metacercariae increase susceptibility of juvenile Chinook salmon to *Flavobacterium columnare* and *Aeromonas salmonicida* infection. These bacteria cause high mortality in juvenile hatchery salmonid populations in the Willamette River, and are a potential threat to wild juvenile salmonids. Juvenile Chinook salmon were first infected with *N. salmincola* through cohabitation with infected freshwater snails, *Juga* spp., then challenged through static immersion with either *F. columnare* or *A. salmonicida*. Cumulative percent mortality from *F. columnare* was higher in *N. salmincola*-parasitized compared to non-parasitized juvenile Chinook salmon. In contrast, cumulative percent mortality from *A. salmonicida* did not differ between *N. salmincola*-parasitized and non-parasitized juvenile Chinook salmon. No mortalities were observed in the *N. salmincola*-only and control groups from either challenge. These results show that a high infection intensity (>200 metacercariae per posterior kidney) of encysted *N. salmincola* metacercariae does not cause mortality alone, but can increase susceptibility to certain bacterial infections in juvenile Chinook salmon.

Distribution and Coinfection of Microparasites and Macroparasites in Juvenile
Salmonids in Three Upper Willamette River Tributaries.

by
Sean Robert Roon

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Presented December 9, 2014
Commencement June 2015

Master of Science thesis of Sean Robert Roon presented on December 9, 2014

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ACKNOWLEDGEMENTS

CONTRIBUTION OF AUTHORS

Dr. Jerri L. Bartholomew provided advice on experimental design, data interpretation, and manuscript editing throughout this thesis. Dr. Julie Alexander provided assistance with experimental design, data interpretation, and statistical analysis for all the research in this thesis. For the first manuscript of this thesis, Michelle Jakaitis, John Rogers, and Craig Banner provided field sampling, fish necropsy, and diagnostic assistance along with data collection. For the second manuscript of this thesis, Dr. Kym Jacobson provided assistance with experimental design and data interpretation.

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CHAPTER 1: INTRODUCTION

DISEASE ECOLOGY

Hosts and parasites live in perpetual tension, under the influence of a myriad of biotic and abiotic factors within their respective ecosystem (Koskella & Lively 2007). Moreover, a shift from equilibrium among host, parasite, and environment often results in increased disease (Hedrick 1998; Daszak 2000; Dobson & Foufopoulos 2001; Johnson & Paull 2011; Okamura & Feist 2011). Specifically, Snieszko (1974) states, "an overt infectious disease occurs when a susceptible host is exposed to a virulent pathogen under proper environmental conditions." Therefore, research on the effects of parasites on wild populations with respect to ecological and environmental factors is critical to providing a mechanistic and predictable understanding of disease dynamics within ecosystems (Daszak 2000).

Ecosystems and parasites

Parasites are conventionally categorized into two groups: microparasites (viruses, bacteria, fungi, and protozoans) and macroparasites (helminths and arthropods, etc.). Microparasites complete a full life cycle or multiply in a definitive host, generally have short generation times, and transmit directly to conspecific hosts (Anderson & May 1979). Macroparasites do not multiply in a definitive host, have longer generation times, and complete a full life cycle through transmission stages (eggs and larvae), which pass into the external environment (Bakke & Harris 1998). Together they represent an integral part of an ecosystem and can act as natural stressors to aquatic organisms (Jacobson *et al.* 2003). Although parasites were once thought to have little influence on an ecosystem, a growing body of research has documented that in many cases, parasites have direct and indirect effects on natural

populations (Anderson & May 1979; Marcogliese & Cone 1997; Lafferty *et al.* 2008).

Parasites can regulate host abundance, which can shift population structure, genetic

variation, and community composition (Hudson 1998; Behringer, Butler & Shields

2006; Decaestecker *et al.* 2013) and host communities may differ based on whether or

not specific parasites are present (Packer *et al.* 2003; Holt & Roy 2007). Furthermore,

host behavior, immune response, and fitness may be associated with parasites

(Lafferty & Morris 1996; Barber, Hoare & Krause 2000; Johnson & Hoverman

2012). For example, habitats with greater parasite diversity have been associated with

greater diversity of immune genes and immune response in threespine sticklebacks

Gasterosteus aculeatus (Kurtz *et al.* 2004; Scharsack *et al.* 2007).

Consequently, it has been hypothesized that diversity begets diversity, as

greater host diversity is commonly associated with greater parasite diversity

(Hechinger & Lafferty 2005; Kamiya *et al.* 2014). Therefore, ecosystems rich with

parasite species may indicate a healthy, functioning and sustainable system (Hudson,

Dobson & Lafferty 2006; Shah *et al.* 2013). Ecological processes are less likely to be

hindered in ecosystems with greater biodiversity as a greater number of functional

traits overlap among species, decreasing the likelihood that sudden environmental

variation will bring the system out of equilibrium (Loreau, Naeem & Inchausti 2002;

Hooper *et al.* 2005). Acidity, eutrophication, pollution, and biodiversity loss have

been associated with decreases in ecosystem health and parasite diversity

(Marcogliese & Cone 1996; Marcogliese & Pietrock 2011; Pietrock & Hursky 2011;

Lafferty 2012). As many parasites depend on the presence of their host, the absence

of a host important in the parasite life cycle will lead to the absence of the parasite

(Lafferty *et al.* 2008). Given that biodiversity loss is a pressing concern for conservation research and management (Cardinale *et al.* 2012), it is crucial to understand the interaction between diversity and disease (Keesing *et al.* 2010).

An ecosystem with high biodiversity can reduce transmission of parasites by decreasing the proportion or abundance of hosts infected, effectively decreasing or diluting the overall disease risk, otherwise known as the dilution effect (Schmidt & Ostfeld 2001). The dilution effect may occur if a greater proportion of resistant hosts or hosts with lower transmission competency are present, decreasing the likelihood of transmitting infectious agents (Keesing, Holt & Ostfeld 2006). Although the dilution effect may not be general to all ecosystems (Wood *et al.* 2014), growing evidence for the important role of parasitic communities in diversity-disease interactions emphasizes the value of community-based approaches to studying disease in the wild (Pasari *et al.* 2013; Johnson *et al.* 2013).

Parasites and salmonids

Micro- and macroparasites can cause acute and chronic disease in captive and wild salmonid populations (Bakke & Harris 1998; Hedrick 1998; Austin & Austin 2012). The infectious hematopoietic necrosis virus (IHNV) endemic to Sockeye salmon (*Oncorhynchus nerka*) can cause significant mortality of wild and captive fry and smolts in freshwater (Williams & Amend 1976). In the Pacific Northwest, cases of population-level disease effects on wild salmonid populations have been associated with *Ichthyophonus hoferi* (Kocan, Hershberger & Winton 2004), *Ichthyophthirius multifiliis* (Traxler, Richard & McDonald 1998), *Apophallus* sp. (Ferguson *et al.* 2011a), *Nanophyetus salmincola* (Jacobson *et al.* 2008), *Ceratonova shasta* (syn.

Ceratomyxa shasta; Hallett *et al.* 2012), and *Parvicapsula minibicornis* (Bradford *et al.* 2010). Observation of disease effects on wild fish populations is difficult because weakened or diseased fish are quickly removed from a natural system by predation or decomposition (La & Cooke 2010). The complex life histories of salmonids make it even further challenging to detect disease epizootics and assess population-level effects (Bakke & Harris 1998; Jacobson *et al.* 2008; Miller *et al.* 2014). Therefore, most cases of disease effects on salmonid populations are first described in captive fish populations. For example, the first case of microsporidiosis caused by *Loma salmonae* infection was reported to cause 10% mortality (~38,000 deaths) in hatchery-reared juvenile Chinook salmon at a large hatchery in Alaska (Hauck 1984).

SALMONIDS IN THE U.S. PACIFIC NORTHWEST

The Pacific Northwest (PNW) is an ecologically and economically important region for hatchery and wild (naturally-reared) salmonids (Friesen, Vile & Pribyl 2007). The Columbia River, the largest river in the PNW region, historically supported the largest Chinook salmon populations in the world (Sheer & Steel 2006). Critical habitat loss, thought to be a consequence of anthropogenic barriers, has led to the decline in numbers of hatchery and wild salmon (*Oncorhynchus* spp.) over the past century in the PNW (Nehlsen, Williams & Lichatowich 1991; Sheer & Steel 2006). The Willamette River (Oregon, USA) is the largest tributary to the Columbia River and its 32,462 km^2 drainage basin provides habitat to threatened Evolutionary Significant Units (ESUs) of spring Chinook salmon *Oncorhynchus tshawytscha* and winter steelhead (rainbow trout) *Oncorhynchus mykiss* (NMFS, 2011). Given that disease is a factor affecting salmonid population dynamics (Arkoosh *et al.* 2004;

Miller *et al.* 2014), elucidating the effects of disease on juvenile salmonids in the Upper Willamette tributaries is critical for conservation and management efforts.

Pathogen transmission between captive and wild fish

Artificial production in public hatcheries has been implemented along PNW rivers to mitigate the decrease in wild salmonid population numbers (McElhany *et al.* 2004). The introduction of artificially-produced or hatchery salmonids can negatively affect wild salmonids through genetic diversity loss, competition, and disease (Youngson & Verspoor 1998). Records suggest an interaction, though direct pathogen transmission from captive fish to wild fish is rarely documented (Johansen *et al.* 2011).

One example of disease transmission to wild salmonids is the introduction of *Aeromonas salmonicida*, the etiological agent of furunculosis, to wild salmonid stocks in Norway from imported, captive rainbow trout in 1964 (Johnsen & Jensen 1994). A second case is also documented in Norway involving the spread of the pathogenic monogenean trematode *Gyrodactylus salaris* being associated with live fish movement from infected hatcheries (Johnsen & Jensen 1991). A survey for similar trematode species *Gyrodactylus colemanensis* and *G. salmonis* found that about 2.3×10^5 live parasites/day were exported from a local hatchery in Nova Scotia, Canada, but patchy distribution in wild fish made it difficult to assess dispersal patterns of the parasite between captive and wild salmonids (You, MacMillan & Cone 2011). Finally, the global emergence of viral hemorrhagic septicemia virus (VHSV) is hypothesized to have been a direct result of several transmission events between captive and wild fish stocks (Kurath & Winton 2011).

Several literature reviews offer concise summaries of transmission events between captive and wild fish, but only speculate as to the effects on wild fish populations (Naish *et al.* 2007; Ford & Myers 2008; Johansen *et al.* 2011; Kurath & Winton 2011). These reviews all conclude that increased wild fish health surveillance of micro- and macroparasites is necessary to provide fundamental data leading towards mechanistic understanding of disease transmission between captive and wild salmonids and the potential effects on wild fish populations (Kent *et al.* 1998; Kent 2011; Ferguson *et al.* 2011b).

Parasite surveys in the Columbia River

In the PNW, many surveys have focused on specific micro- and macroparasites in the Columbia River basin. Pacha and Ordal (1970) detected *Flavobacterium columnare*, the causative agent of columnaris disease, in adult salmonids migrating upstream in the Columbia River with the incidence of *F. columnare* increasing with water temperature. Several detections were also reported in juvenile spring Chinook salmon from two tributaries, Yakima and Snake Rivers. Isolation of *F. columnare* from suckers (*Catostomus* spp.), carp (Cypriniformes), and whitefish (*Prosopium* spp.) provided circumstantial evidence for non-salmonid disease reservoirs. *Renibacterium salmoninarum,* the causative agent of bacterial kidney disease (BKD), was detected in approximately 20% of salmonids collected in the lower Columbia River during a study to assess the effects of BKD on juvenile salmonid populations (Sanders *et al.* 1992). Van Gaest *et al.* (2011) surveyed hatchery-reared Chinook salmon at various points along their out-migration through the Columbia River to observe if microparasite prevalence differed between fish

barged through the dams and fish migrating in-river. Salmon kidneys were screened using molecular methods and the most prevalent microparasites were *R. salmoninarum* and IHNV. Additionally, there were infrequent detections of infectious pancreatic necrosis virus (IPNV) and five Gram-negative bacteria (*Flavobacterium psychrophilum, Aeromonas hydrophila, Aeromonas salmonicida, Vibrio anguillarum,* and *Yersinia ruckeri*) throughout all sampling sites. Microparasite prevalence was greater in fish that were barged through the dams than in fish left to migrate in-river, suggesting transmission occurs during the barging process (Van Gaest *et al.* 2011).

Parasite surveys in PNW coastal estuaries

Other parasite surveys have focused on PNW coastal estuaries. A survey of wild juvenile coho (*Oncorhynchus kisutch*) and Chinook salmon in PNW estuaries found that the most prevalent macroparasite for both species was *Nanophyetus salmincola,* which was detected at all twelve estuaries surveyed with a prevalence range of 33% - 100% (Arkoosh *et al.* 2004). The most prevalent microparasites were *R. salmoninarum,* erythrocytic necrosis virus (or erythrocytic inclusion body syndrome; EIBS) and three Gram-negative bacteria (*Vibrio anguillarum, Yersinia ruckeri,* and *A. salmonicida*). The prevalence of *N. salmincola* and *R. salmoninarum* was higher in Chinook compared to coho salmon. A survey of metazoan parasites along the Oregon coast found 21 different parasite species in coho salmon (Ferguson *et al.* 2011b). The most common macroparasites were *N. salmincola, Apophallus* sp., and *Myxobolus insidiosus*. In general, fry and parr were more intensely infected than smolts; this temporal decline in parasite burdens (number of parasites/infected fish) may have been related to parasite-associated mortality.

Parasites of Willamette River salmonids

Based on over 30 years of fish examinations, ODFW fish health specialists report a variety of metazoan parasites detected in fishes in the upper Willamette River tributaries including: *Nanophyetus salmincola, Diplostomum* spp., *Crepidostomum* spp., *Gyrodactylus* spp., *Dactylogyrus* spp., *Capillaria salvelini, Myxobolus insidiosus, Myxobolus* spp., *Chloromyxum* spp., *Henneguya* spp., *I. multifiliis, Trichodina* spp., *Trichodinella* spp., and gill copepods *Salmincola californiensis* (Mann *et al.* 2010). A range of parasites have also been documented in returning adult Chinook salmon in the Willamette River. One technical report lists the following parasites detected in adults collected throughout the Willamette River mainstem and tributaries: *N. salmincola, Apophallus* sp., *Echinochasmus* spp., *P. minibicornis, Myxobolus* spp. in the brain, *C. shasta, R. salmoninarum,* and *A. salmonicida* (Schreck *et al.* 2013). Similarly, another study comparing the sensitivity and specificity of histology to traditional diagnostic tests found that returning adults in the Willamette River were commonly infected with *A. salmonicida, R. salmoninarum, C. shasta, P. minibicornis, Myxobolus* spp. in the brain, and *N. salmincola* (Kent *et al.* 2013). Moreover, the authors occasionally detected anisakine nematode larvae, adult cestodes (likely *Eubothrium* sp.), *Myxidium* sp., *L. salmonae*, *I. multifiliis,* and gill copepods (Kent *et al*. 2013).

Five microparasites cause significant mortality in hatchery and wild salmonids in this region: *Aeromonas salmonicida, Flavobacterium columnare, Flavobacterium psychrophilum, Renibacterium salmoninarum,* and IHNV (Tony Amandi, ODFW fish health specialist, *pers. comm.*). The Gram-negative bacteria *A. salmonicida, F.*

columnare, and *F. psychrophilum* typically cause acute mortality but can lead to chronic disease, whereas the Gram-positive bacteria *R. salmoninarum* primarily causes chronic disease in salmonids (Austin & Austin 2012). IHNV is a rhabdovirus that typically causes acute mortality in juvenile salmonids, but can lead to chronic mortality in adults (LaPatra 1998). These microparasites are known to spread through waterborne transmission, linked to high mortality events in salmonids, present in returning adult salmonids, and can be detected in subclinical carriers (Miller *et al.* 2014). Epizootics of these pathogens are driven by environmental factors such as temperature, fish crowding, and poor water quality (e.g. high organic matter content, low dissolved oxygen and pH; Wakabayashi 1991; LaPatra 1998; Austin & Austin 2012). For example, higher temperatures result in a higher growth rate of *F. columnare* and can lead to higher mortality in salmonids (Holt *et al.* 1975; Pulkkinen 2010).

Parasite distribution has been documented in returning adult Chinook salmon in the upper Willamette River tributaries, whereas, there have been no published micro- or macroparasite surveys of wild juvenile salmonids in these watersheds. Therefore, I examined juvenile salmonids from upper Willamette River tributaries for parasites and present the results in the second chapter of this thesis.

Parasite-associated mortality

Many metazoan parasites may not be directly linked to host mortality, but can indirectly affect host fitness and survival (Jacobson *et al.* 2003; Ferguson *et al.* 2012). For example, studies have observed microsporidian infection to reduce body condition and decrease body mass in American winter flounder (*Pseudopleuronectes*

americanus) and Atlantic cod (*Gadus morhua*) (Cali *et al.* 1986; Khan 2005). Furthermore, increasing microsporidian *Enterocytozoon salmonis* intensity (number of parasites per infected host) was negatively associated with the humoral immune response in Chinook salmon (Wongtavatchai, Conrad & Hedrick 1995).

Based on the negative effects that parasites may have on fish fitness and survival and chronic or persistent infection with certain parasite species, methods have been developed to estimate parasite-associated mortality in wild fish populations (Lester 1984). For example the reduction of parasite prevalence or dispersion (variance to mean ratio of parasite abundance) over time indicates parasite-associated mortality. Declines in prevalence and variance to mean ratio of *N. salmincola* over time have been used as evidence for early marine mortality of juvenile coho salmon (Jacobson et al. 2008). Furthermore, parasite infection intensity follows a negative binomial distribution across a population, where the majority of hosts harbor few or no parasites and heavily parasitized individuals make up a smaller proportion (Ferguson *et al.* 2012). Given that heavily infected individuals are more prone to mortality, truncation of a predicted negative binomial distribution of *Apophallus* sp. intensity provided evidence for parasite-associated mortality in wild juvenile coho salmon in the West Fork Smith River, OR, as there were fewer heavily infected individuals than predicted (Ferguson *et al.* 2011a).

Nanophyetus salmincola

As shown in the surveys mentioned above, *N. salmincola* is a common macroparasite found in PNW salmonids. Salmonids are a second intermediate host for this digenean trematode. Free-living cercariae are released from the freshwater

intermediate snail host, *Juga* spp. snails, penetrate and encyst as metacercariae in soft

tissues, predominantly in the posterior kidney and posterior musculature near the

caudal peduncle (Michael Kent, *pers. comm.*), where they remain encysted until the

fish is consumed by a definitive host, a mammal or piscivorous bird (Fig. 1.1). The

parasite is found along the PNW coast from northern California to the Puget Sound of

Washington as determined by the *Juga* spp. (*Juga silicula* or *J. plicifera*) snail

distribution (Furnish 1990). Temperature is the most likely driver of *N. salmincola*

release from snails; low temperatures inhibits cercarial release causing a seasonal

pattern of *N. salmincola* cercariae release from snails starting in spring and ending in

the winter (Amandi 1977). Interestingly, a study comparing production of juvenile

Chinook salmon in heated and unheated artificial streams observed lower infection

rates of *N. salmincola* in the heated stream, indicating that elevated temperatures may

decrease *N. salmincola* transmission (Bisson 1974).

      Baldwin et al. (1967) observed mortality of six different types of salmonids

within 24 h of infection with controlled doses of cercariae. However, most of these

fish were quite small (29 – 42mm, fork length) and were non-endemic species to the

PNW, where the parasite naturally occurs. Experimental exposures of cercariae to

fish over longer periods of time, allowed for accumulation of parasite numbers that

would be lethal if exposed in a 24 h period without any effect on survival (Millemann

& Knapp 1970). Conclusions from these experiments were that *N. salmincola*

metacercariae are not highly pathogenic, but the mechanical injury sustained from

cercarial migration are pathogenic, specifically to small fish (Bennington & Pratt

1960, Millemann & Knapp 1970).
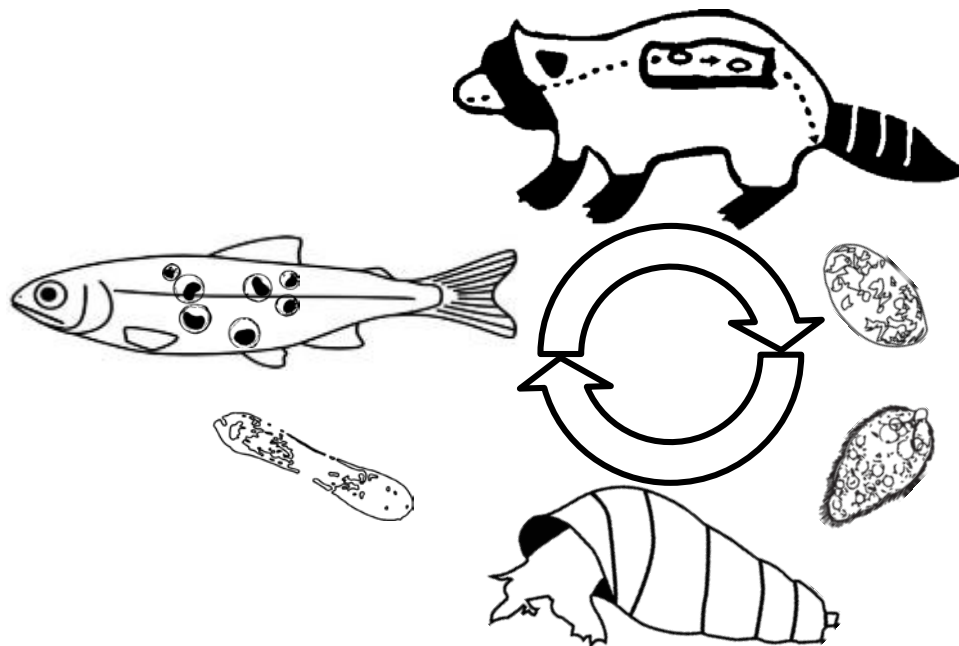
Figure 1.1. The life cycle of *Nanophyetus salmincola* requires an intermediate freshwater *Juga* spp. snail to release cercariae that penetrate and encyst as metacercariae in a second intermediate salmonid host.

Further field and laboratory studies have shown that encysted *N. salmincola* metacercariae can have sub-lethal effects on juvenile salmonid fitness and survival. Experimental exposure of yearling Chinook to *N. salmincola* showed that parasitized fish did show an increase in immunoglobulin and a decrease in saltwater osmoregulation ability (Foott et al. 1997). Laboratory experiments with coho salmon smolts found that fitness parameters such as growth, swimming stamina, and ATPase activity (an enzyme linked to osmoregulation) were negatively associated with *N. salmincola* parasitism (Ferguson *et al.* 2012). Jacobson et al. (2003) demonstrated that *N. salmincola* can decrease immune function and disease resistance in juvenile Chinook salmon. Mortality from *V. anguillarum* was higher in groups of fish

parasitized with *N. salmincola* compared with non-parasitized groups. Furthermore, the authors observed that the combination of a natural stressor and an anthropogenic stressor had a greater negative effect on the B cell responses of juvenile Chinook salmon than either stressor alone (Jacobson *et al.* 2003). Histopathology indicates mild, chronic inflammation around *N. salmincola* metacercariae (Ferguson *et al.* 2010), therefore, negative effects from this parasite may be related to chronic stress and its suppression of immune function (Maule *et al.* 1989). Consequently, these sub-lethal effects may explain the association between *N. salmincola* and early marine mortality of juvenile coho salmon observed during surveillance of parasite intensity within wild populations (Jacobson et al. 2008).

PARASITE COINFECTION

Across ecosystems it is common to observe a single host infected with many different types of parasites (Raso 2004; Johnson & Hoverman 2012). Therefore, focus on a single host-parasite interaction are less ecologically relevant as parasite interactions (coinfections) are known to influence parasite prevalence, distribution, and intensity as much as age or season (Telfer *et al.* 2010). As species interactions are important at a macro scale, so are interactions at a micro scale (Poulin 2011). Coinfection interactions can determine within-host distribution and abundance of parasites through direct competition for host resources or through indirect facilitation by modulating host immune response (Graham 2008). An indirect interaction can occur between macro- and microparasites, which can facilitate microparasite success within the host. In humans, it has been shown that helminth infection can affect the severity of malaria through an immune-mediated response (Hartgers &

Yazdanbakhsh 2006). In free-ranging African buffalo, one study demonstrated that infection patterns of *Mycobacterium bovis*, an emerging chronic bacterial disease, were most likely driven by coinfection interactions with gastrointestinal nematodes. The authors conclude that increased mortality and transmission efficiency rates of *M. bovis* in coinfected individuals was due to an immune trade-off resulting from an active Th2 response suppressing the Th1 pathway (Jolles *et al.* 2008).

Coinfection in fish

Though the precise mechanism underlying macroparasite-microparasite interactions within aquatic organisms is unknown, it is evident from field and laboratory studies that within-host interactions are important to consider within an aquatic ecosystem (Johnson & Hoverman 2012). It is possible that chronic stress or suppressed immune function from macroparasites may facilitate microparasite infection (Tort 2011). Laboratory experiments have shown that parasite coinfections involving various parasite species in channel catfish (*Ictalurus punctatus*) can lead to higher host mortality (Labrie *et al.* 2004; Xu *et al.* 2012; Shoemaker *et al.* 2012). Another possible mechanism for macroparasite-microparasite interactions may be from mechanical damage, as macroparasite attachment and penetration can enhance *Aeromonas hydrophila* and *F. columnare* infection (Pylkkö *et al.* 2006; Bandilla *et al.* 2006).

The route of infection and tissue parasitized may determine if a macroparasite affects disease susceptibility. Suomalainen et al. (2005) tested whether a variety of stressors, including: exposure to an eye trematode *Diplostomum spathaceum*, netting, pH change, oxygen depletion, and abrasion could reinstate a *F. columnare* epizootic

in fish that had survived a previous *F. columnare* epizootic. Neither macroparasite nor stress treatments induced clinical columnaris signs. A subsequent *F. columnare* immersion challenge did induce columnaris disease, but there were no significant differences observed between *D. spathaceum* exposed tanks and controls. The lack of coinfection interaction could be due to proposed immune-hidden status of the eye where *D. spathaceum* metacercariae encyst. Therefore, facilitation of a microparasite by an existing macroparasite infection may depend on the particular parasite species and site of infection. The third chapter of this thesis describes a laboratory experiment designed to investigate if *N. salmincola* can affect disease susceptibility of juvenile Chinook in a freshwater system.

RESEARCH OBJECTIVES

The objectives of this thesis are to document the prevalence and distribution of specific microparasites and metazoan parasite communities in juvenile salmonid populations in upper Willamette tributaries and assess how coinfection may affect the disease ecology of juvenile salmonids. I achieve this objective through a series of field surveys and laboratory experiments. The following chapters are manuscripts that address parts of this thesis.

- Chapter 2 documents the prevalence of five specific microparasites and metazoan parasite communities in juvenile salmonids from three upper Willamette tributaries.

- Chapter 3 presents two laboratory experiments that investigate how encysted *N. salmincola* metacercariae affect juvenile Chinook salmon susceptibility to two pathogenic bacteria.

- Chapter 4 concludes with an overall summary and discusses the principle findings of this research with respect to disease dynamics, community ecology, and conservation of wild salmonid populations.

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CHAPTER 2: SURVEY OF MICRO- AND MACROPARASITES IN WILD JUVENILE CHINOOK SALMON AND RAINBOW TROUT IN THREE UPPER WILLAMETTE RIVER TRIBUTARIES

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Abstract

Wild fish populations are typically infected with a variety of micro- and macroparasites that may affect fitness and survival, however, there is little published information on parasite distribution in wild juvenile salmonids in the upper tributaries of the Willamette River, OR. The objectives of this survey were to document (1) the distribution of select microparasites in wild salmonids and (2) the prevalence, geographical distribution, and community composition of metazoan parasites infecting these fish. From 2011-2013, we surveyed 279 Chinook salmon *Oncorhynchus tshawytscha* and 149 rainbow trout *O. mykiss* for one viral (IHNV) and four bacterial (*Aeromonas salmonicida, Flavobacterium columnare, Flavobacterium psychrophilum*, and *Renibacterium salmoninarum*) microparasites known to cause mortality of fish in Willamette River hatcheries. The only microparasite detected was *Renibacterium salmoninarum*, causative agent of bacterial kidney disease, which was detected at all three sites. We identified 23 metazoan parasite taxa in a subset of 220 Chinook salmon and 130 rainbow trout examined by wet mount preparation. Nonmetric multidimensional scaling of metazoan parasite communities reflected a nested structure with trematode metacercariae being the basal parasite taxa at all three sites. The freshwater trematode *Nanophyetus salmincola* was the most common macroparasite observed at all three sites. Additional parasites commonly observed included: *Apophallus* sp., *Sanguinicola* sp., *Myxobolus insidiosus,* and *Salmincola californiensis*. In 2012, parasite richness in juvenile Chinook salmon was similar at all three sites, but parasite richness in juvenile rainbow trout was lowest at the warmest site.

Introduction

The Willamette River (Oregon, USA) is an ecologically and economically important habitat for hatchery and naturally-reared (wild) salmonids (Friesen et al. 2007), containing Evolutionary Significant Units (ESUs) of spring Chinook salmon *Oncorhynchus tshawytscha* and winter steelhead (rainbow trout) *Oncorhynchus mykiss* that are both listed as threatened under the Endangered Species Act (NMFS 2011). Disease can have a significant effect on dynamics of wild salmonid populations (Arkoosh et al. 2004, Miller et al. 2014), and may be a contributing factor to the decline in numbers of hatchery and wild salmonids over the past decades (Sheer and Steel 2006). In the Pacific Northwest, cases of population-level disease effects on wild salmonid populations have been associated with *Ichthyophonus hoferi* (Kocan et al. 2004), *Ichthyophthirius multifiliis* (Traxler et al. 1998), *Apophallus* sp. (Ferguson *et al.* 2011a), *Nanophyetus salmincola* (Jacobson *et al.* 2008), *Ceratonova shasta* (syn. *Ceratomyxa shasta*; Hallett *et al.* 2012), *Myxobolus cerebralis* (Koel et al. 2006) and *Parvicapsula minibicornis* (Bradford *et al.* 2010). To better understand population-level effects, surveillance is key to establishing information on micro- and macroparasite distribution and is an important component of conservation and management plans for threatened salmonid stocks (Kent et al. 1998, Ferguson et al. 2011b). As parasite-associated mortality in outmigrating juvenile fish can contribute to reduced recruitment and adult returns (Hallett et al. 2012), it is important to document parasite prevalence and distribution in the upper reaches where juvenile salmonids rear. However, for the Willamette River, surveys have focused on

returning adults (Keefer et al. 2010, Kent et al. 2013), and there is little data on micro- and macroparasites in wild juvenile salmonid in the upper tributaries of the system.

Few parasites are directly linked to host mortality, but many can indirectly affect host fitness and survival by hindering growth, swimming stamina, and immune function of salmonids (e.g. Jacobson et al. 2003, Ferguson et al. 2011a, 2012). Measuring these effects in the wild is difficult given that diseased fish are quickly removed from natural systems by predation or decomposition (Mesa et al. 1998, Johansen et al. 2011, Sikes and Paul 2013, Miller et al. 2014). Lester (1984) reviewed methods for estimating mortality in wild fishes associated with chronic, subclinical effects on fish and parasite distribution in a host population. He concluded that if a parasite persists and is not cleared by the host, reduction of prevalence or dispersion (variance to mean ratio of parasite abundance; VMR) over time indicates parasite-associated mortality. For example, declines in prevalence and VMR of the digenean trematode *Nanophyetus salmincola* over time have been used as evidence for early marine mortality of juvenile coho salmon *Oncorhynchus kisutch* (Jacobson et al. 2008).

Even though individual parasite species may affect host populations, studies on single host-parasite interactions are of limited ecological relevance as natural systems harbor multiple hosts and parasites (Keesing et al. 2010). Changes to an ecosystem affect both host and parasite community composition (Vidal-Martinez et al. 2010, Pietrock and Hursky 2011), which in turn can affect disease dynamics (Johnson and Hoverman 2012) and overall host fitness and survival (Lafferty and

Morris 1996, Scharsack et al. 2007). Therefore, placing parasites into a community ecological context is necessary for better understanding disease and will complement studies on single host–pathogen interactions (Johnson et al. 2013).

This survey was conducted to provide data on micro- and macroparasites that may have negative effects on threatened wild juvenile salmonids in three upper Willamette River tributaries. The objectives of this survey were to document (1) the distribution of select microparasites in wild salmonids and (2) the prevalence, geographical distribution, and community composition of metazoan parasites infecting these fish. Here, we report survey results and discuss differences in prevalence of micro- and macroparasites and variation among study sites.

Methods

Site descriptions

Wild fish were collected from sites on three upper Willamette River tributaries (Fig. 2.1): Middle Fork Willamette River near Dexter Ponds Rearing Facility (MFW; 43º55'48.53", -122º48'55.41"), McKenzie River near Leaburg and McKenzie Hatcheries (MCK; 44º07'33.97", -122º36'51.93"), and South Santiam River near South Santiam Hatchery (SS; 44º24'45.97", -122º41'07.95"). All sites are located in proximity (< 1km) to a fish hatchery or rearing facility and directly downstream of a dam (Dexter, Leaburg, and Foster Dam, respectively). Sites were proximal to hatchery facilities to examine the potential for pathogen transmission between hatchery and wild populations. Leaburg and McKenzie fish hatcheries were treated as one site due to their close vicinity along the river. Water temperature and river discharge data were recorded by United States Geological Survey (USGS)

monitoring stations in each tributary (Table 2.2). The USGS stations (MFW, USGS

site #14150000, Willamette River rkm 324; MCK temperature, USGS site

#14162500, McKenzie River rkm 76.8; MCK discharge, USGS site #14163150,

McKenzie River rkm 60.2; SS, USGS site #14162500, South Santiam River rkm

59.6) are all located within 1km of each site, with the exception of the MCK

temperature station, which is located within 15km. Elevation profiles were recorded

for each site using Google Earth (Table 2.2).

Fish collections

From 2011-2013, the Oregon Department of Fish and Wildlife (ODFW)

collected wild juvenile salmonids. Field sampling was performed opportunistically

throughout June – November and efforts were characterized by season: summer (June

– September) and autumn (October – November). These periods were chosen because

the higher river temperatures are linked to increased epizootic risk (Wedemeyer

1996). In general, fish were collected using an electroshock raft and seine nets

starting at a facility effluent and moving approximately 3 km downstream. Fish were

euthanized by an overdose of buffered tricaine methanesulfonate (10 ml/L of MS-222

stock solution, Argent Chemical Laboratories) and were transported on ice in coolers

within 24 h of capture. Fish were assumed wild if the adipose fin was unclipped

(adipose fin clipping is a common marking technique for hatchery fish in Oregon).

Wild salmonids were classified as juveniles based on fork length (FL) measurements

taken from published length-frequency distributions of steelhead and Chinook

migrants (Ward et al. 1989, Billman et al. 2014).

In 2011, fish were collected from the MCK and MFW sites during the autumn period. In 2012, fish were collected at all three sites during the summer period, additional autumn sampling was completed in November at the MCK site. In 2013, fish were collected at MCK and MFW sites during summer and autumn. Further fish samples were collected at any site during a disease epizootic affecting the nearby facility. In Nov. 2012, fish were collected at the MFW site during an epizootic of *A. salmonicida* and *F. columnare* at the nearby Dexter Ponds rearing facility, and again in Sept. 2013, during an epizootic of *F. columnare*.

Diagnostic procedures

The following microparasites were selected for this study based on the likelihood of distribution in Oregon watersheds (ODFW Senior Fish Health Specialist, Tony Amandi *pers. comm.*) and association with mortality events in hatchery and wild salmonid populations (Table 2.1): *Aeromonas salmonicida, Flavobacterium columnare, Flavobacterium psychrophilum*, *Renibacterium salmoninarum,* and Infectious Hematopoietic Necrosis Virus (IHNV). Fish collected at each site were examined for the presence of specific microparasites and metazoan parasites within 48 h of collection. Protocols for detection of select microparasites followed standard methods described in the American Fisheries Society Fish Health Section Blue Book (AFS-FHS 2010). In brief, kidney subsamples were cultured for bacterial isolation on tryptic soy agar (TSA, BD Biosciences) and tryptone yeast extract salts agar (TYES: 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2; Holt et al. 1989). Plates were incubated at 18˚C for 7 days. *A. salmonicida, F. columnare,* and *F. psychrophilum* were

presumptive identified using morphologic and biochemical characteristics. Presence

of *R. salmoninarum* was determined using a direct fluorescent antibody test (DFAT)

or an enzyme-linked immunosorbent assay (ELISA). For smaller fish, kidney smears

were examined using a DFAT (samples include: 2011 MCK; 2012 MCK summer, SS

summer (Chinook only); 2013 MCK). If a sufficient kidney mass was available

(minimum 0.7g), an ELISA was performed to detect *R. salmoninarum* antigen

(samples include: 2012 MFW, MCK autumn, SS summer (rainbow trout only)). Gill,

kidney, and spleen tissues from 5 fish of same species were pooled for IHNV

detection. Samples were processed and inoculated onto two separate cells lines:

epithelioma papulosum cyprini (EPC), and Chinook salmon embryo (CHSE-214).

Cells were monitored at 3, 7, 11, and 14 d intervals for cytopathic effects (CPE).

Metazoan parasite identification

A general parasite examination was performed on a subset of fish samples

collected. The external surface, mouth, gills, skin (scale scrape), skeletal muscle (filet

section), eye, posterior kidney, gall bladder, intestine, brain tissue (in 2013 only) were

examined for metazoan parasites. In 2013, the posterior half of kidneys were

collected from all fish in Stomacher® bags (Seward Ltd.) and frozen at -20˚C for later

quantification of *N. salmincola* infection intensity (number of parasite/posterior

kidney) as *N. salmincola* metacercariae can be encyst throughout soft tissues of fish,

but aggregate in the posterior kidney (Jacobson et al. 2003). *N. salmincola*

metacercariae are identified by the presence of an opaque posterior excretory bladder

(Hoffman 1999). Fish were examined for metazoan parasites within 48 h of

collection, or were flash frozen by submerging whole, ventrally-incised bodies in -

80˚C ethanol (95%) for 2 min. After 2 min, the liquid was decanted and individual samples stored at -20˚C until examination. Parasites were detected by examining wet mounts of fish tissues at $100\times$ magnification using a compound light microscope and identified to lowest possible taxa using morphological traits and tissue distribution, referring to keys of known fish parasites (Hoffman 1999).

Terms are defined according to Bush et al. (1997): 'prevalence' is the percent of a host sample infected, 'intensity' is the number of a particular parasite species in an infected host, 'mean intensity' is the average intensity of a particular parasite species among the infected hosts in a sample, 'mean abundance' is the number of a particular parasite species in a host sample divided by the total number of hosts examined, including uninfected hosts, and 'infracommunity' is the community of all parasite species in a single host. Parasite infracommunity richness ("parasite richness" hereafter) is defined as the number of unique parasite taxa present in an individual fish. For 2013 samples, parasite dispersion among hosts was determined by the variance to mean ratio ($s^2/\bar{x}$), calculated by dividing the sample variance by the sample mean abundance.

Statistical analysis

For each site, Fisher's exact tests were used to compare prevalence of individual microparasites between fish species. Within each fish species, Fisher's exact tests with Bonferonni correction were used to compare prevalence of micro- and macroparasites among sites and between seasons. Mean parasite richness was compared among three sites using a one-way ANOVA. Significance was set at $P < 0.05$. Only fish that were fully examined for parasites were included for parasite

assemblage analyses. Prevalence of *R. salmoninarum* in samples assayed with DFAT were not compared with samples assayed with ELISA, as these assays have different detection sensitivities (Meyers et al. 1993, Elliott et al. 2013).

Metazoan parasite community analysis

Because it was not always possible to identify parasites to species, parasites were categorized based on taxa, tissue infected, and transmission pathway (Table 2.4). Trophic transmission requires the consumption of infected host and waterborne is transmission through a free-living stage. The presence-absence of these parasite taxa groups were used for the analysis of metazoan parasite assemblage structure. Nonmetric multidimensional scaling (NMDS) was completed on the Euclidean distance matrix in PC-ORD 6 (McCune and Mefford 2011) and used to compare parasite communities among sites, season, and species in each survey year. The same NMDS method was used to compare parasite communities of juvenile Chinook salmon at MCK during the autumn sampling period over all three survey years. The Euclidean distance measure was chosen to be able to compare the presence – absence of parasite taxa, including fish samples with no detected parasites. Joint plots between matrices of parasite communities and environmental variables of site and fish sample were used to interpret environmental factors associated with NMDS ordination (Table 2.2). All parasite species were included for calculation of parasite richness, but rare parasites (< 1% prevalence in total sample population) were excluded prior to NMDS ordination to improve resolution of parasite community structure using multivariate analyses.

Results

Microparasite Survey

From 2011 – 2013, a total of 279 wild juvenile Chinook salmon (89.2 mm ±

1.3; mean FL ± SE) and 149 wild juvenile rainbow trout (108.2 mm ± 3.0) were

examined for the five specific microparasites. No gross signs of clinical disease were

observed for any salmonid specimens. In 2011, none of the target microparasites were

detected. In 2012 and 2013, *R. salmoninarum*, the causative agent of bacterial kidney

disease, was the only target microparasite detected (Table 2.3). In 2012, the

prevalence of *R. salmoninarum* by ELISA in Chinook salmon ranged among sites

from 0-30% (mean prevalence, 3%) and in rainbow trout ranged from 0-50% (mean

prevalence, 4%). *R. salmoninarum* was not detected in Chinook salmon by DFAT.

Infection prevalence in rainbow trout ranged among sites from 0-5% (mean

prevalence, 2.5%), as one rainbow trout was found *R. salmoninarum*-positive by

DFAT at the MFW site. In 2013, no fish were assayed by ELISA, and the prevalence

of *R. salmoninarum* by DFAT in Chinook salmon ranged from 0-39% (mean

prevalence, 18.7%), but was not detected in rainbow trout. From 2011-2013, no

differences in *R. salmoninarum* prevalence by either assay were detected among sites

or between fish species, although *R. salmoninarum* prevalence (by DFAT) in

Chinook salmon at the MCK site was significantly (Fisher's Exact Test, $P = 0.019$)

higher in the summer compared to the autumn sampling period. The majority of

ELISA-positive fish had low ELISA optical density (OD) values (0.100-0.200),

indicating light infections. Similarly, the majority of DFAT-positive fish were scored

as light infections (1-10 bacteria per field) based on the number of fluorescing bacteria per $1,000\times$ microscopic field.

Surveys downstream of facilities experiencing epizootics

In Nov. 2012, Chinook salmon ($n = 7$) and rainbow trout ($n = 27$) were collected at the MFW site while fish in the Dexter Ponds rearing facility were experiencing an epizootic of *A. salmonicida* and *F. columnare*. Neither microparasite was detected in samples collected. In Sept. 2013, Chinook salmon ($n = 1$), rainbow trout ($n = 1$), and sculpin *Cottus* spp. ($n = 11$) were collected at the MFW site coinciding with the occurrence of a high mortality event linked to *F. columnare* at the Dexter Ponds facility. *F. columnare* was not detected in either salmonid, but *F. columnare* was isolated from 3 of 11 sculpin (Table C.2).

Metazoan parasites

From 2011-2013, kidneys from a total of 251 Chinook salmon and 131 rainbow trout were examined for *N. salmincola* metacercariae, with a full parasite examination performed on a subset of 220 Chinook salmon and 130 rainbow trout. General parasitological examinations identified 23 parasite taxa in both salmonid species, and these are summarized in Table 2.4. The occurrence of common metazoan parasite species is summarized in Table 2.5. The prevalence of the most common parasite *N. salmincola* was high at all three sites in both Chinook salmon (33-100%) and rainbow trout (47-100%). In 2012, prevalence of *N. salmincola* was significantly (Fisher's Exact Test, $P < 0.017$) lower at the MFW site for both Chinook salmon (33%) and rainbow trout (47%) compared to MCK (97%, 100%; Chinook and rainbow prevalence, respectively) and SS (90%, 100%; Chinook and rainbow

prevalence, respectively) sites during the summer sampling period. At MFW, prevalence of *N. salmincola* in rainbow trout in summer (47%) was significantly lower (Fisher's Exact Test, $P < 0.025$) compared to autumn (82%). In 2013 at MCK, most *N. salmincola* infections in Chinook salmon were of low intensity (1-25 metacercariae/posterior kidney), although values ranged to 234 metacercariae/ posterior kidney (Fig. 2.2) and the VMR increased from summer (30.5) to autumn (76.3).

Additional parasites commonly detected were *Apophallus* sp., *Myxobolus insidiosus*, *Sanguinicola* spp., and *Salmincola californiensis* (Table 2.5). *Apophallus* sp. were identified as small metacercariae, absent of collar spines, and often had a crease in the middle, although, some metacercariae were difficult to distinguish between *Apophallus* sp. and *Echinochasmus milvi* and were labeled as unidentified metacercariae. *Myxobolus insidiosus* spores were identified from pseudocysts comprised of numerous spindle-shaped cysts in myocytes. *Sanguinicola* spp. were identified by the presence of eggs and miracidia in the gills. Rare parasite detections (found in $< 1\%$ of all samples) of *Deropegus aspina* in the intestine, *Hexamita* spp. in the intestine, *Epistylis* spp. on the skin, glochidia (bivalve clam larvae) on gill filaments, and *Chloromyxum myxidium* in the posterior kidney were also observed (Table 2.4). For brain tissue examined in 2013, *Myxobolus* sp. was detected in one Chinook salmon.

For metazoan parasite richness, sites were compared during the 2012 summer sampling period as this represents the most comprehensive dataset. In 2012, mean parasite richness in rainbow trout was significantly ($F_2 = 33.3$, $P < 0.0001$) lower at

MFW, whereas there was no difference between mean parasite richness at SS and MCK (Fig. 2.3A). Mean parasite richness in Chinook salmon was similar ($F_2 = 1.51$, $P = 0.228$) among all three sites (Fig. 2.3B).

The parasite community reflected a nested structure with low parasite richness associated with the presence of species of trematode metacercariae, and high parasite richness associated with the additional presence of species of myxozoans, gill miracidia from *Sanguinicola* spp., adult Digenean trematodes, nematodes, copepods, cestodes, microsporidians, glochidia, ciliates, and flagellates (Table 2.4). For the 2012 summer period, NMDS of rainbow trout parasite communities provided a two-dimensional solution (stress = 10.28) demonstrating some separation of fish among sites based on the nested strucuture described above. A joint plot overlay indicated a negative association of ordination scores along Axis 1 between parasite richness and temperature with a weak association of ordination scores along Axis 2 between mean seasonal discharge and elevation (Fig. 2.4). For the same period, NMDS of Chinook salmon parasite communities (stress = 13.52) did not demonstrate separation among sites, but MFW samples were similarly nested in SS and MCK samples based on infection of trematode metacercariae, with the greater spread of the samples from the latter sites indicating the presence of less common parasite taxa groups (Fig. 2.5). In 2012, NMDS indicated modest separation (stress = 2.93) between Chinook salmon and rainbow trout at MCK, ordination scores were associated with parasite richness and FL (Fig. 2.6). From 2011-2013, parasite richness in Chinook salmon did not differ ($F_2 = 0.55$, $P = 0.461$) among years at the reference site, MCK. NMDS of

Chinook salmon provided a two-dimensional solution (2D stress = 8.30) indicating

parasite community structure remained constant throughout all three years (Fig. 2.7).

Discussion

Detection of bacterial and viral microparasites

From 2011-2013, there were no detections of *A. salmonicida*, *F. columnare*,

F. psychrophilum, or IHNV in wild juvenile Chinook salmon or rainbow trout

collected in three upper Willamette River tributaries. There was 11.4% prevalence of

R. salmoninarum in Chinook salmon and 7.6% in rainbow trout collected from these

sites. Surveys of juvenile Chinook salmon in the Columbia River also reported *R.*

salmoninarum prevalences of 93% (Arkoosh et al. 2004) and 32% (Van Gaest et al.

2011) using more sensitive molecular methods. Other surveyed microparasites (*A.*

salmonicida, *F. psychrophilum,* and IHNV) were not detected using the same culture-

based diagnostic methods as the current study (Arkoosh et al. 2004) or present at low

(< 5%) prevalence using molecular methods (Van Gaest et al. 2011). A survey of

ocean-caught fish in the coastal waters of British Columbia, Canada found

R. salmoninarum prevalences of 58% in Chinook, 42% in coho, and 6% in sockeye

salmon *O. nerka* (Kent et al. 1998), and similar prevalences of 11% (Banner et al.

1986) to 29% (Sandell et al. 2014) are reported in juvenile Chinook salmon in coastal

waters of Oregon and Washington. For returning adult Chinook salmon collected

throughout the Willamette River mainstem and tributaries, Kent et al. (2013) report a

23% prevalence of *R. salmoninarum* but also found 54% prevalence of

A. salmonicida using culture-based detection methods. It is possible some of these

microparasites are maintained in a system through carrier fish (LaPatra 1998, Austin

and Austin 2012) or in the environment (Kunttu et al. 2012). Therefore, chronic

infections, such as *R. salmoninarum*, may be detected more readily as the other microparasites (Gram-negative bacteria and INHV) generally cause acute disease only when certain environmental conditions are met, causing ephemeral incidence of epizootics (Decostere et al. 1999, Ogut and Reno 2004).

The observed interannual variability of *R. salmoninarum* prevalence in juvenile Chinook salmon at the MCK site from 0% in 2011 to 39% in 2013 and the seasonal decline from 39% in summer to 17% in autumn in 2013 is similar to variation reported in other studies. Sandell et al. (2014) similarly reported high interannual variation in *R. salmoninarum* prevalence in subyearling Chinook salmon shifting in surveys conducted off the coast of Oregon and Washington in 1999 (14%), 2000 (65%), 2001-2003 (28%) and 2004 (6%). A seasonal decline in *R. salmoninarum* prevalence between early (36-66%) and late (9-19%) season cohorts was also observed in a 2007 survey of out-migrating juvenile Chinook salmon originating from two hatcheries in the Columbia River (Van Gaest et al. 2011). This seasonal decline may be indicative of recovery from infection (Cvitanich 2004, Sandell et al. 2014) or lower transmission to fish later in the season (Van Gaest et al. 2011). Alternatively, a decrease in prevalence may be a result of diseased individuals being removed through disease-induced mortality or increased susceptibility to predation (Mesa et al. 1998). Seasonal variation between *R. salmoninarum* prevalence was also observed in 2012; however this was possibly due to differences in detection sensitivities between ELISA and DFAT (Meyers et al. 1993). Consequently, prevalence was not statistically compared between samples assayed with different tests.

Metazoan parasites

The metazoan parasites detected in the current study were similar to those detected by ODFW in wild fish examinations conducted over the past 30 years (Mann et al. 2010) and in wild juvenile coho salmon in the West Fork (WF) Smith River, OR (Ferguson et al. 2011b). In the latter study, *N. salmincola, Apophallus* sp., and *Myxobolus* spp. were also commonly detected. Prevalence of *N. salmincola* in the current study in Chinook salmon (33-100%) and rainbow trout (47-100%) was similar to *N. salmincola* prevalence observed in juvenile coho (73-98%) and Chinook salmon (33-100%) in Oregon and Washington estuaries (Arkoosh et al. 2004). Kent et al. (2013) report a similar 90% prevalence of *N. salmincola* but with a higher mean of 512 (range, 1-7,156) metacercariae/g of kidney in returning adult Chinook salmon in the Willamette River compared to juveniles in the current study. Additionally, common detection of *Apophallus* sp., *Echinochasmus* sp., and *Myxobolus* spp. in the brain with occasional detections of anisakine nematode larvae, adult cestodes (likely *Eubothrium* sp.), *Myxidium* sp., *L. salmonae*, and gill copepods *S. californiensis* in returning adults are similar to many of the parasites observed in the current study (Schreck et al. 2013, Kent et al. 2013).

The metazoan parasites *N. salmincola, M. insidiosus,* and *Apophallus* sp. commonly detected in this survey can have subclinical effects on fitness and survival of wild juvenile salmonids (Jacobson et al. 2008, Ferguson et al. 2011a, 2012). The decrease in the prevalence and VMR of *N. salmincola* was used to provide evidence for parasite-associated mortality of juvenile coho salmon off the coast of Oregon and Washington (Jacobson et al. 2008). However, similar to the findings of Ferguson et

al. (2011a), the current study found the VMR increased from summer to autumn in juvenile Chinook salmon, supporting the concept that the parasite is only associated with increased mortality after salmon enter the ocean.

Metazoan parasite community structure varied along an apparent gradient of parasite richness, which reflected a nested structure in which the low richness site (MFW) was a subset of richer parasite assemblages (SS and MCK). Trematode metacercariae represented basal parasite taxa present at low richness sites, whereas high richness sites were represented by species from less commonly detected parasite taxa (myxozoans, adult trematodes, nematodes, and cestodes) in addition to the basal parasite taxa of trematode metacercariae. Nested structures of parasite communities have been documented in marine fishes (Rohde et al. 1998) and amphibians (Johnson and Hoverman 2012). Several abiotic and biotic factors, including host abundance and temperature, are associated with parasite prevalence, richness, community structure in zooplankton (Ebert et al. 2001), freshwater snails (Altman and Byers 2014), and marine fishes (Poulin and Rohde 1997). In the current study, parasite richness was negatively associated with increasing river temperatures, as the higher temperature (13-15˚C) at the MFW site compared to SS and MCK (9-12˚C) coincided with the decreased detection of myxozoan and trophically-transmitted parasite taxa. These higher temperatures may have affected parasite richness by acting on the free-living stages of these parasites or the composition and abundance of invertebrate and fish hosts (Pietrock and Marcogliese 2003, Marcogliese 2005). Furthermore, the lower parasite richness at MFW may reflect habitat quality, as past reports of low wild Chinook salmon abundance in the Middle Fork Willamette River have been

attributed to the low habitat quality in this watershed (McElhany et al. 2004), supporting the concept that parasites may be used to indicate ecosystem health or sustainable functioning (Shea et al. 2012, Lafferty 2012).

Differences in parasite assemblage ordinations between salmonid species was associated with fish length and the presence of trophically transmitted parasites. As fish length is generally correlated with fish age (Zajanc et al. 2013), increased contact time with parasites throughout a host's lifespan in-river is likely to result in more diverse parasite communities (Zander et al. 2000). Furthermore, a difference between trophically transmitted parasites presence is most likely related to differences in foraging behavior and habitat selection as both can influence parasite distribution, prevalence, and intensity (Marcogliese and Cone 1997, Hall et al. 2007, Rodnick et al. 2008).

Micro- and macroparasite distribution in the Willamette River

Although *A. salmonicida*, *F. columnare*, *F. psychrophilum*, or IHNV were not dectected in juvenile salmonids in this survey, the high *A. salmonicida* prevalence in returning adults (Kent et al. 2013) and the isolation of *F. columnare* from resident sculpin at the MFW site demonstrates that these microparasites are present in this system and indicates that non-salmonid species may be a reservoir. This increased prevalence is likely due to the immunocompromised state of spawning adult Chinook salmon, as these fish are destined to die after spawning (Keefer et al. 2010). These fish also pass through the mainstems of the Columbia and Willamette River, which may contribute to increased prevalence through waterborne transmission (Keefer et al. 2010, Van Gaest et al. 2011) and may encounter other parasites not present in the

upper tributaries. For example, the metazoan parasities *I. multifiliis*, *C. shasta,* and *P. minibicornis* were not detected in any wild salmonids in the current study. Nor were they detected in WF Smith River juvenile coho salmon (Ferguson et al. 2011b), with the exception of one detection of *I. multifiliis*. These are of concern because they have been associated with high mortality in salmonid populations (Traxler et al. 1998, Hallett et al. 2012, Bradford et al. 2010). However, *C. shasta* and *P. minibicornis* were commonly detected in adult Chinook salmon in the Willamette River (Kent et al. 2013). Infections of these myxozoan parasites in adult Chinook salmon and the higher *N. salmincola* intensity and bacterial loads may indicate that immunocompromised adults are drivers of certain micro- and macroparasite life cycles in this watershed (Kent et al. 2014). Conversly, given the adults pass through the mainstem to the upper tributaries, the lack of detection in juveniles may indicate that the invertebrate hosts necessary to complete the life cycle are absent in the upper tributaries (Hallett et al. 2012).

It is possible that bacterial, viral, and other metazoan parasites were present at a low prevalence but were not detected because of our limited sample numbers. Some metazoan parasites (e.g. *P. minibicornis*, *C. shasta*, *Nucleospora salmonis*, certain external parasites) may not have been easily detected using the methods in this study (Docker et al. 1997, Kent et al. 1998a, 2013), but the methods used were consistent between sites. Freezing samples was necessary in order to be able to examine some fish before decomposition. This process damages parasite structure, and therefore, some adult digenean trematodes and nematodes were unidentified due to lack of structure resolution. Furthermore, detection of flagellate and ciliate parasites was

most likely hindered throughout the survey as these parasites quickly deteriorate after the fish is taken from the water, and freezing would destroy these parasites. Therefore, some microparasites or metazoan parasites may be present within this system, despite a lack of detection.

Summary

In this survey, target microparasites were not detected (*A. salmonicida*, *F. columnare*, *F. psychrophilum*, and IHNV) or were detected at low prevalence (*R. salmoninarum*) in wild juvenile Chinook salmon and rainbow trout in the three Upper Willamette tributaries surveyed. Similar to the lighter macroparasite infection intensities in the upper WF Smith River (Ferguson et al. 2011b), the Willamette River tributaries may represent refuge from high micro- and macroparasite burdens for rearing juveniles. Parasite richness differed among sites, with the lowest parasite richness at the MFW site, coinciding with the warmest water temperatures and poorest salmon habitat quality. Further studies should focus on understanding how parasite richness and community structure relate to fish communities and habitat quality and incorporate histology and molecular methods to increase sensitivity and broaden detection of parasites (Ferguson et al. 2011b). Given that parasite distributions and community diversity are often linked to ecosystem health (Marcogliese 2005, Pietrock and Hursky 2011) and infection risk (Johnson et al. 2013), community-based approaches are essential to studying disease in the wild. Determining general relationships between micro- and macroparasite distributions, host diversity, and environmental variables will elucidate how biodiversity-disease interactions may affect wild juvenile salmonids.

Acknowledgements

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Table 2.1. Specific microparasites surveyed in juvenile Chinook and rainbow trout collected in upper Willamette River tributaries.

Target Microparasite	Agent	Disease	Epizootic/ high loss association	Present in returning adult salmonids	Carrier State	Temperature Response
Aeromonas salmonicida	Gram-negative bacteria	Furunculosis	(Johnsen and Jensen 1994)	(Kent et al. 1998)	(Bullock and Stuckey 1975)	(Groberg Jr. et al. 1978)
Flavobacterium columnare	Gram-negative bacteria	Columnaris	(Pacha and Ordal 1970)	(Becker and Fujihara 1978)	(Suomalainen et al. 2005)	(Holt et al. 1975)
Flavobacterium psychrophilum	Gram-negative bacteria	Cold-water disease	(Cipriano et al. 1996)	(Kent 2011, Miller et al. 2014)	(Madsen et al. 2005)	(Holt et al. 1989)
Renibacterium salmoninarum	Gram-positive bacteria	Bacterial kidney disease	(Sanders et al. 1992)	(Fryer and Sanders 1981)	(Fryer and Sanders 1981)	(Jones et al. 2007)
Infectious Hematopoietic Necrosis Virus	Rhabdovirus	IHNV	(Williams and Amend 1976)	(LaPatra 1998)	(LaPatra 1998)	(LaPatra 1998)

Table 2.2. Physical characteristics of sample sites in the Willamette River during the summers of 2011 – 2013. Mean seasonal discharge values are listed with the coefficient of variation (CV) in parentheses. Sites are abbreviated as follows: McKenzie River (MCK), Middle Fork Willamette River (MFW), and South Santiam (SS).

| | | | | Temperature | | | | | | |
| | | | | Seasonal | | | | During sampling | | |
Year	Season and site	Elevation (m)	Mean Seasonal Discharge -(CV) (m^3/s)	Min.	Max.	Mean	CV	Min.	Max.	Mean
	Summer (Jun – Sep)									
2011	MCK	215.8	92.7 (20.5)	8.5	15.2	11.6	13.0	11.0	14.2	12.5
2012	MCK	215.8	89.4 (13.0)	8.6	15.7	11.8	13.3	10.4	15.6	12.8
	MFW	191.4	61.2 (14.6)	12.7	17.5	14.7	5.9	14.4	16.1	15.1
	SS	157.3	31.2 (26.8)	9.7	13.2	11.7	5.6	11.2	12.8	11.9
2013	MCK	215.8	68.0 (12.5)	8.3	16.1	12.4	13.1	10.6	15.8	13.0
	MFW	191.4	66.9 (23.9)	12.6	17.5	15.0	6.2	15.7	16.6	15.9
	Autumn (Oct – Nov)									
2011	MCK	215.8	89.0 (15.0)	5.5	11.9	9.1	16.2	6.7	8.4	7.7
2012	MCK	215.8	118.5 (42.6)	6.3	12.0	9.2	13.9	6.9	7.6	7.2
	MFW	191.4	88.3 (26.8)	11.3	15.7	13.9	7.7	13.6	13.8	13.8
2013	MCK	215.8	111.0 (31.3)	5.2	11.2	8.9	13.1	5.2	7.0	6.2

Table 2.3. Prevalence of *Renibacterium salmoninarum* detected in juvenile Chinook salmon (Chs) and rainbow trout (Rbt) from 2011-2013. The values are the number of positive/the total number analyzed; positives are bold with prevalence of infected individuals in parentheses. Years are separated by season: summer (Jun – Sep) and autumn (Oct – Nov). Comparisons between *R. salmoninarum* prevalence

Site and species of sample	Year					
	2011		2012		2013	
	Summer	Autumn	Summer	Autumn	Summer	Autumn
MCK						
Chs	0/11[+]	0/48[+]	0/64[+]	3/10† (30%)	24/62[+] (39%[a])	8/48[+] (17%[b])
Rbt	-	0/29[+]	1/19[+] (5%)	1/2† (50%)	0/3[+]	0/1[+]
MFW						
Chs	-	-	1/6† (17%)	0/7†	0/1[+]	-
Rbt	-	-	1/32† (3%)	0/27†	0/1[+]	-
SS						
Chs	-	-	0/35[+]	-	-	-
Rbt	-	-	1/35† (3%)	-	-	-

†Positive samples for *R. salmoninarum* were detected by ELISA, as a sufficient kidney mass (> 0.7g) was collected from these samples.

[+] Positive samples for *R. salmoninarum* were detected by DFAT, a test used to detect *R. salmoninarum* presence in the small kidney mass (< 0.7g) collected from these samples.

*Prevalence between seasons at a given site and year with different lowercase superscript letters are significantly different ($P < 0.05$)

- Indicates no samples were collected

Table 2.4. Parasites grouped by taxa, tissue(s) infected, and transmission pathway to fish. Trophic transmission is consumption of infected host and waterborne is transmission through a free-living stage. Trematode species are separated by life stage. The overall prevalence represents the combined prevalence from 2011-2013 in Chinook salmon and rainbow trout.

Parasite Taxon	Overall prevalence (%)	Transmission to fish	Tissues Found
Trematode metacercariae	**89†**		
Nanophyetus salmincola	85	Waterborne	Gill, kidney, muscle, heart, intestine
Apophallus sp.	27	Waterborne	Gill, muscle
Echinochasmus milvi	6	Waterborne	Gill
Unidentified metacercariae	16	Waterborne	Gill
Myxozoans	**32†**		
Myxobolus insidiosus	18	Waterborne	Muscle
Myxobolus fryeri	6	Waterborne	Muscle (nerves)
Myxobolus squamalis	16	Waterborne	Skin (scale pockets)
Myxobolus sp.	< 1	Waterborne	Brain (only examined in 2013)
Gill miracidiae	**22†**		
Sanguinicola sp.	22	Waterborne	Gill
Adult trematodes	**17†**		
Crepidostomum spp.	8	Trophic	Intestine
Plagioporus shawi	2	Trophic	Intestine
Deropegus aspina	< 1	Trophic	Intestine
Unidentified Digenea	8	Trophic	Intestine
Nematodes	**16†**		
Rhabdochona spp.	9	Trophic	Intestine
Truettadacnitis spp.	3	Trophic	Intestine
Unidentified Nematode	5	Trophic	Intestine
Copepods	**15†**		
Salmincola californiensis	15	Waterborne	Gill, external (fins, mouth, operculum)

Table 2.4 – continued.

Parasite Taxon	Overall prevalence (%)	Transmission to fish	Tissues Found
Cestodes	**2**†		
Proteocephalus spp.	1	Trophic	Body cavity, intestine, pyloric caecae
Diphyllobothrium spp.	1	Trophic	Intestine, pyloric caecae
Microsporidians	**< 1**†		
Loma salmonae		Waterborne	Gill
Glochidia	**< 1**†		
Unidentified bivalve larvae		Waterborne	Gill
Ciliates	**< 1**†		
Epistylis spp.		Waterborne	External (skin, fins)
Flagellates	**< 1**†		
Hexamita spp.		Waterborne	Intestine

† The bold value represents the prevalence of that taxa group, indicating the prevalence of sampled fish infected with at least one parasite species from that taxa group.

Table 2.5. Prevalence of most common parasites (>15% prevalence) detected in juvenile Chinook salmon and rainbow trout from 2011-2013. Values are the number of positive/the total number analyzed; positives are bold with range and mean intensity of infected individuals in parentheses. Years are separated by season: summer (Jun – Sep) and autumn (Oct – Nov).

Parasite, year, and species	Site				
	MCK		MFW		SS
	Summer	Autumn	Summer	Autumn	Summer
Nanophyetus salmincola (metacercariae/fish)					
2011					
Chinook salmon	**10/11**	**29/37 (1-105, 12)**	-	-	-
Rainbow trout	-	**30/30**	-	-	-
2012					
Chinook salmon	**53/56**	**6/10**	**2/6**[*]	0/4	**17/19**
Rainbow trout	**19/19**	**2/2**	**15/32**[*]	**22/27**	**16/16**
2013					
Chinook salmon	**55/57 (5-150, 33)**	**25/31 (1-234, 73)**	**1/1 (7)**	-	-
Rainbow trout	**3/3 (58-798, 428)**	**1/1 (783)**	**1/1 (9)**	-	-
Apophallus sp. (metacercariae/gill filament)					
2011					
Chinook salmon	0/11	0/37	-	-	-
Rainbow trout	-	0/30	-	-	-
2012					
Chinook salmon	**14/56**	**1/10**	**1/6**	0/4	**8/19**
Rainbow trout	**11/19**	**1/2**	**9/32**	**6/27**	**7/16**
2013					
Chinook salmon	**12/29 (1-10, 4)**	**7/31 (1-6, 4)**	**1/1 (1)**	-	-
Rainbow trout	**2/3 (11-30, 21)**	0/1	**1/1 (2)**	-	-
Myxobolus insidiosus (pseudocyst/fish)					
2011					
Chinook salmon	0/11	**20/37 (10-750, 321)**	-	-	-
Rainbow trout	-	**9/30**	-	-	-
2012					
Chinook salmon	**5/56**	**3/10**	0/6	0/4	0/19
Rainbow trout	**9/19**	0/2	0/32	**1/27**	**1/16**
2013					
Chinook salmon	0/29	**8/31 (13-760, 364)**	0/1	-	-
Rainbow trout	0/3	0/1	0/1	-	-
Sanguinicola sp. (miracidia/gill filament)					
2011					
Chinook salmon	**1/11**	**4/37**	-	-	-
Rainbow trout	-	**7/30**	-	-	-
2012					
Chinook salmon	0/56	**2/10**	0/6	0/4	**2/19**
Rainbow trout	**14/19**	0/2	**4/32**	**2/27**	**7/16**
2013					
Chinook salmon	**2/29 (80-120, 100)**	**5/31 (21-174, 78)**	0/1	-	-
Rainbow trout	**2/3 (40-130, 85)**	0/1	0/1	-	-
Salmincola californiensis (parasite/fish)					
2011					
Chinook salmon	0/11	**5/37**	-	-	-
Rainbow trout	-	**6/30**	-	-	-
2012					
Chinook salmon	**1/56**	**4/10**	0/6	0/4	0/19
Rainbow trout	**2/19**	**1/2**	0/32	0/27	**4/16**
2013					
Chinook salmon	0/29	**7/31 (1-5, 3)**	0/1	-	-
Rainbow trout	0/3	0/1	**1/1 (1)**	-	-
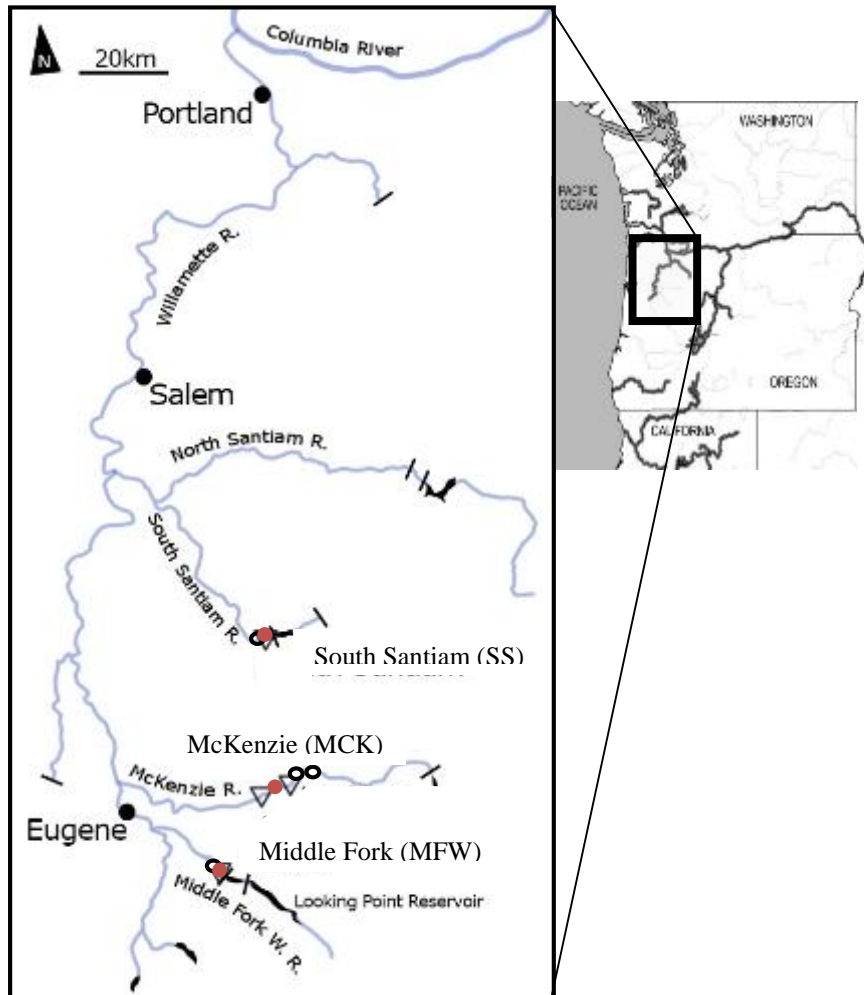
Figure 2.1. Map of the Willamette River Basin. Collection sites are labeled with red circles, fish hatcheries are labeled with open triangles, and USGS stations labeled with open circles. Black bars represent locations of dams.

a)
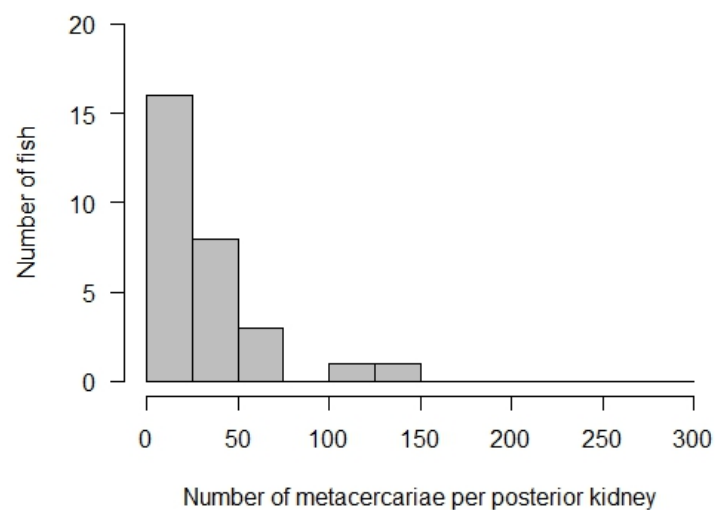
b)
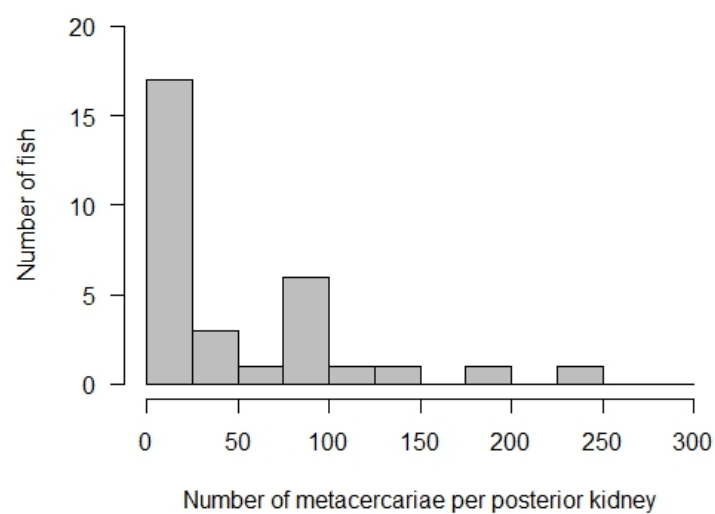
Figure 2.2. *Nanophyetus salmincola* infection intensity in wild juvenile Chinook salmon collected at the McKenzie River (MCK) site in 2013: (a) summer (*n* = 57) and (b) autumn (*n* = 31).
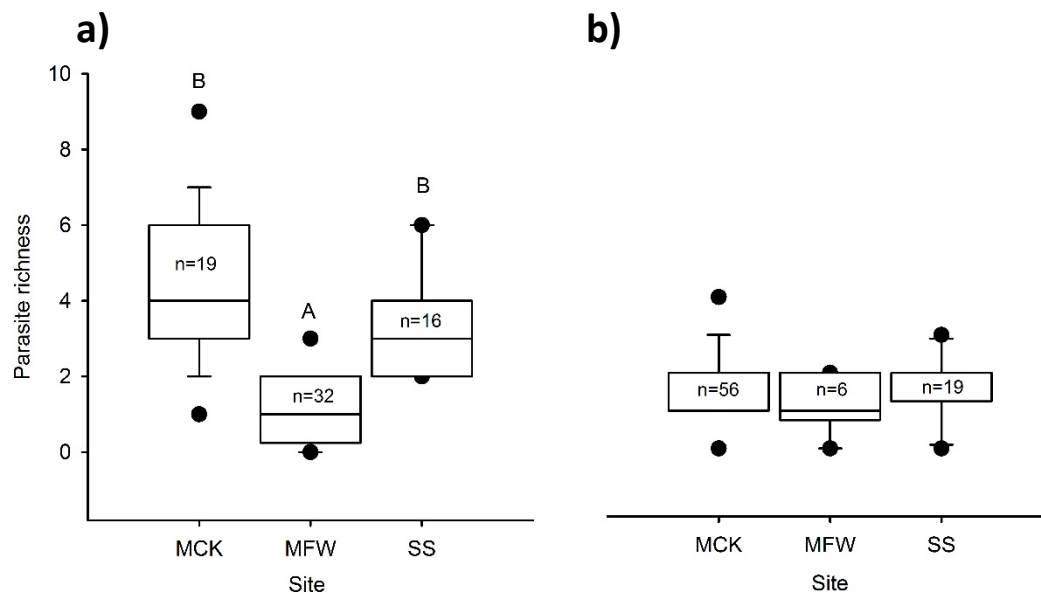
Figure 2.3. Parasite richness in two wild juvenile salmonid species at three sites, McKenzie River (MCK), Middle Fork Willamette River (MFW), and South Santiam (SS), sampled during the 2012 summer period. (a) Mean parasite richness in rainbow trout *Oncorhynchus mykiss* was significantly lower at the MFW site. Sites with different uppercase letters are significantly different ($P < 0.05$). (b) Mean parasite richness in Chinook salmon *O. tshawytscha* did not differ among sites.
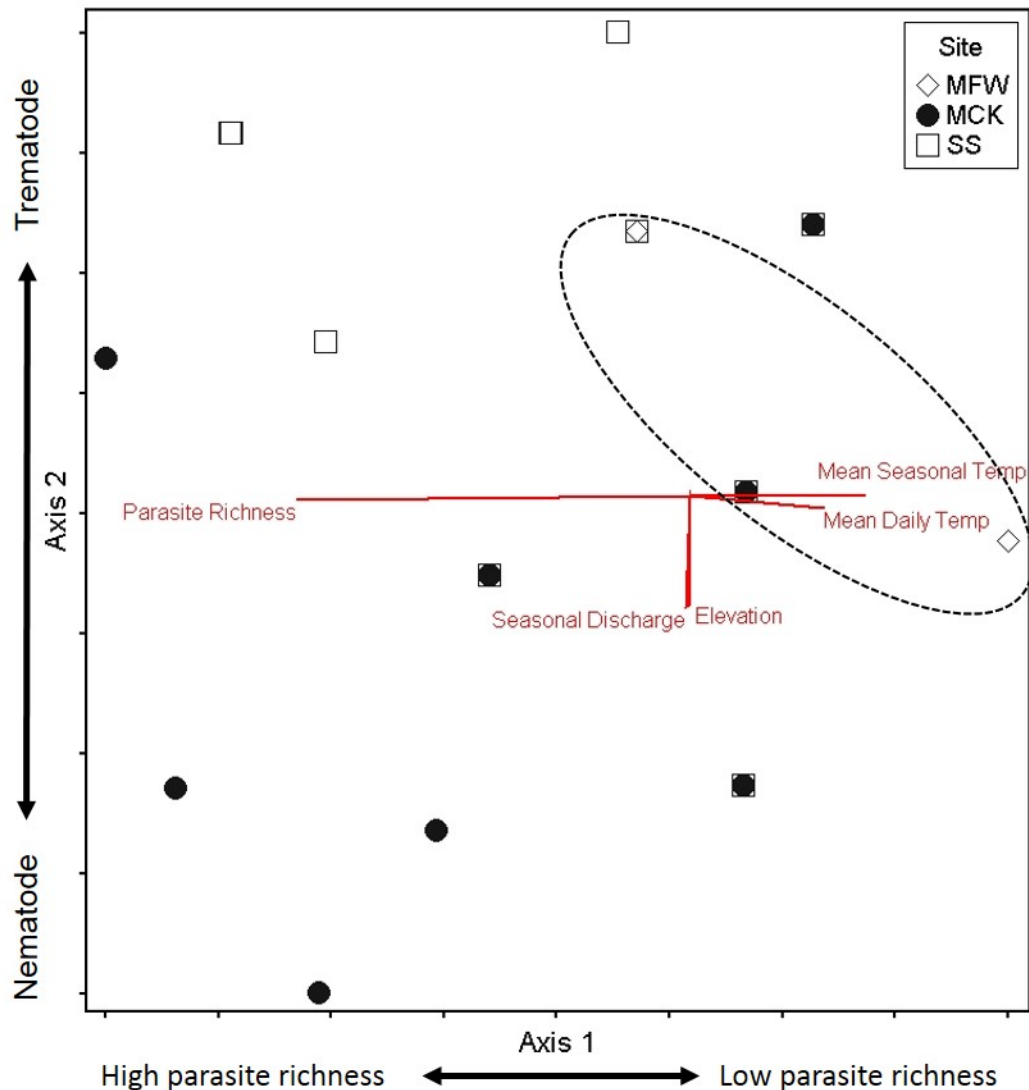
Figure 2.4. Nonmetric multidimensional scaling (stress = 10.28) of parasite assemblage data from rainbow trout sampled during the 2012 summer period at three sites. The MFW samples are grouped within the dashed line. Overlap between sites occurs with infection of trematode metacercariae, and separates along Axis 1 based infection of less commonly observed parasites, associated with increasing parasite species richness and decreasing temperature. Further separation within groups along Axis 2, appears to be driven by trophically-transmitted parasite infections with adult trematodes at the top of the axis and nematodes towards the bottom. The joint plot indicates a weak association of Axis 2 ordination with mean seasonal discharge and elevation.
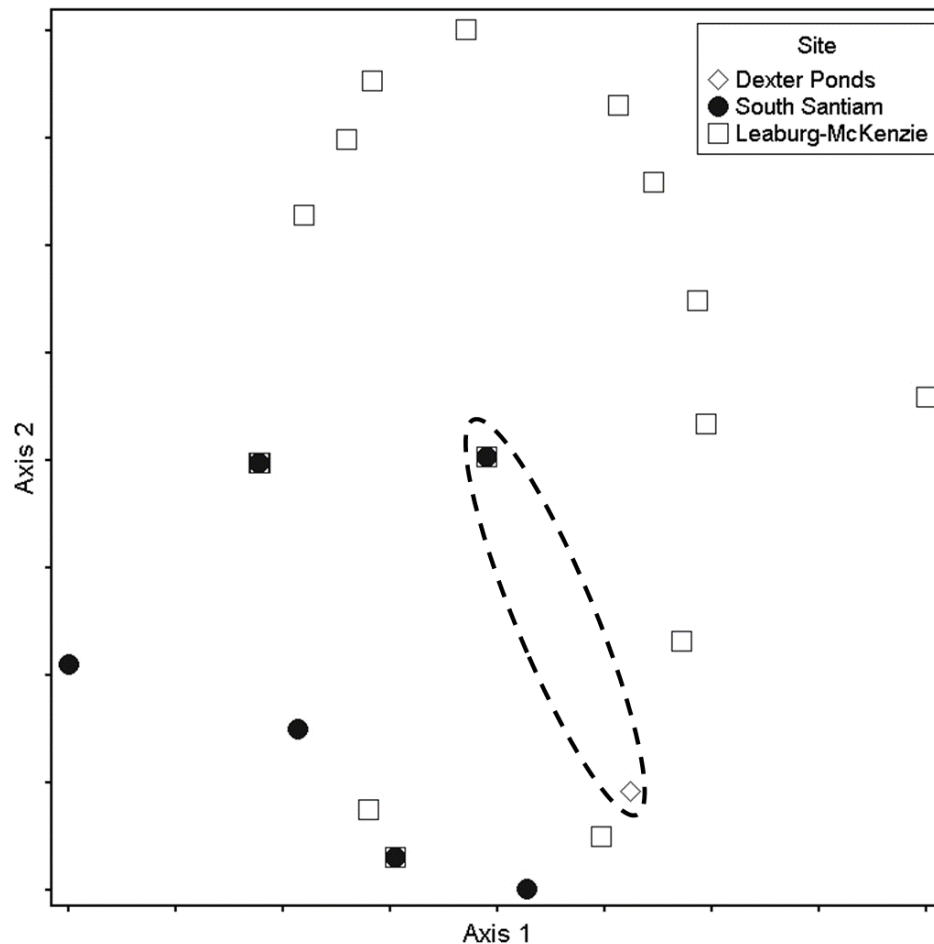
Figure 2.5. Nonmetric multidimensional scaling (stress = 13.52) of parasite assemblage data from Chinook salmon sampled during the 2012 summer period at three sites. The MFW samples are highlighted within the dashed line. Overlap between sites occurs with infection of trematode metacercariae, and separates along Axis 1 based infection of less commonly observed parasites, with the left associated with blood trematode miracidia and adult trematode infection and right associated with copepod infection. Further separation within samples along Axis 2 is associated with myxozoans at the top of the axis and nematodes towards the bottom.
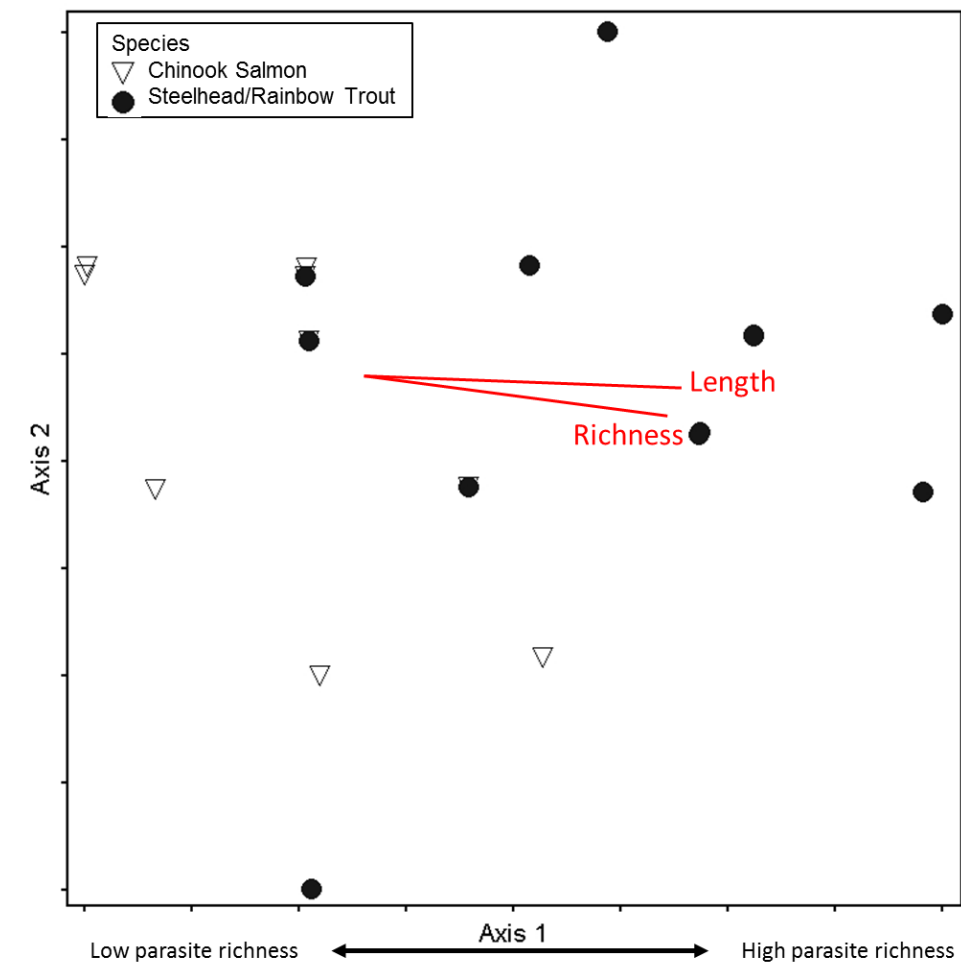
Figure 2.6. Nonmetric multidimensional scaling (stress = 2.93) of parasite assemblage data from juvenile Chinook salmon and rainbow trout during the 2012 summer period from the MCK site. Overlap between species occurs in samples infected with trematode metacercariae, and separates along Axis 1 based on infection of less commonly observed parasite taxa, appearing to be associated with an increase in species richness and fork length. Further separation within groups along Axis 2 is associated with infection of different trophically-transmitted parasites, samples with nematodes ordinate towards the top and samples with adult trematodes ordinate towards the bottom.
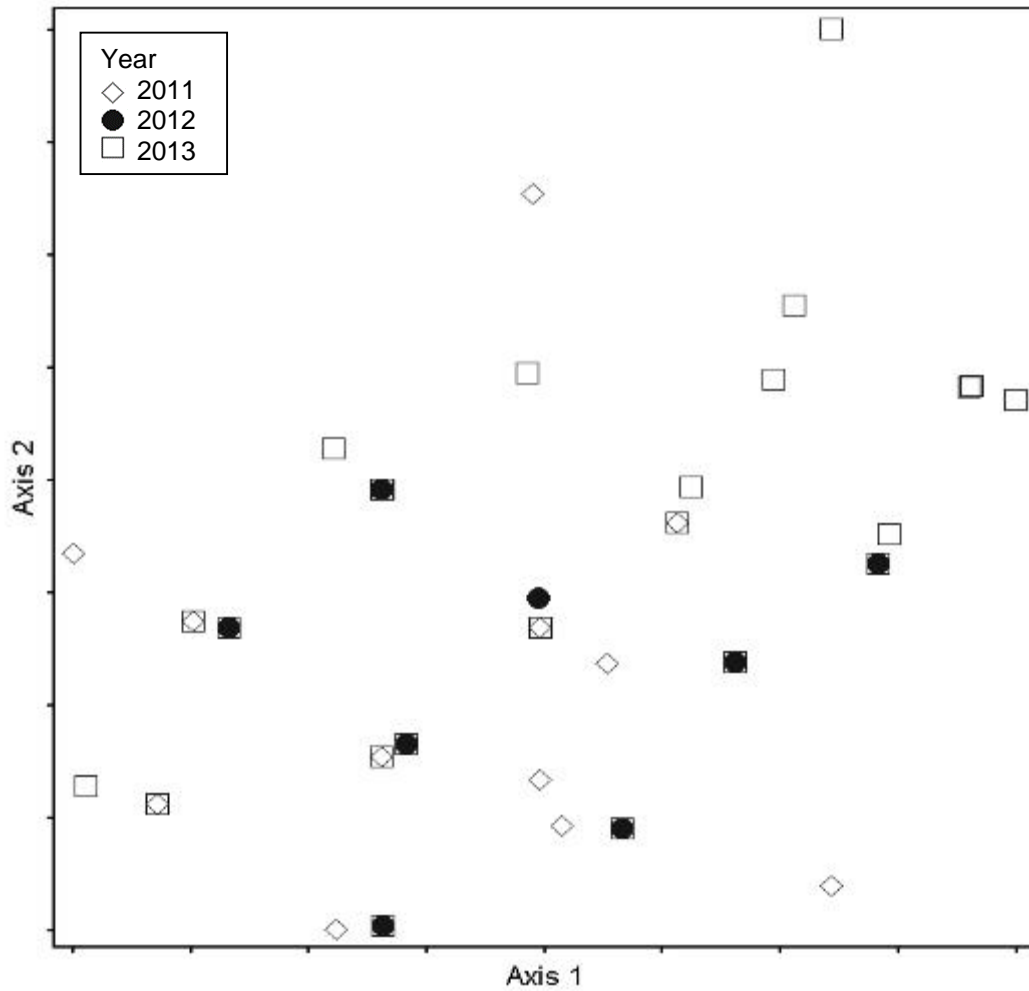
Figure 2.7. Nonmetric multidimensional scaling (stress = 8.30) of parasite communities from wild juvenile Chinook salmon at MCK from 2011-2013 during the autumn period. No distinct separation is indicated from the ordination, indicating parasite communities remained constant throughout the survey.

CHAPTER 3: COINFECTION EFFECTS OF *NANOPHYETUS SALMINCOLA*
CHAPIN AND TWO PATHOGENIC BACTERIA ON JUVENILE CHINOOK
SALMON, *ONCORHYNCHUS TSHAWYTSCHA* (WALBAUM)

Sean R. Roon, Julie D. Alexander, Kym C. Jacobson, & Jerri L. Bartholomew

Abstract

The freshwater trematode *Nanophyetus salmincola* (Chapin) has been shown to impair salmonid immune function and disease resistance to a marine pathogen. We conducted disease challenges to evaluate whether encysted *N. salmincola* metacercariae increase susceptibility of juvenile Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum), to *Flavobacterium columnare* and *Aeromonas salmonicida* infection. These bacteria cause high mortality in captive juvenile salmonid populations in the Pacific Northwest of North America and are a potential threat to wild juvenile salmonids. Juvenile Chinook salmon were first infected with *N. salmincola* through cohabitation with infected freshwater snails, *Juga* spp., then challenged with either *F. columnare* or *A. salmonicida*. Cumulative percent mortality from *F. columnare* was higher in *N. salmincola*-parasitized compared to non-parasitized juvenile Chinook salmon. In contrast, cumulative percent mortality from *A. salmonicida* did not differ between *N. salmincola*-parasitized and non-parasitized groups. No mortalities were observed in the *N. salmincola*-only and control groups from either challenge. Our results show that a high mean intensity (>200 metacercariae per fish) of encysted *N. salmincola* metacercariae does not cause mortality alone, but can increase susceptibility to freshwater bacterial infection in juvenile Chinook salmon.

Introduction

In a natural system, fish populations can be regulated or limited by microparasites (viruses, bacteria, and protozoans) and macroparasites (helminths and arthropods, etc.; Anderson & May 1979; Hudson 1998; Hershberger, Hart, Gregg,

Elder & Winton 2006). Individual wild fish commonly host multiple parasites (Marcogliese & Cone 1997) and interactions between parasite types (coinfection) may yield different results than those predicted by single infection studies (Pedersen & Fenton 2007). Effects of coinfection on mortality, transmission, and recovery rates may have far-reaching effects on disease dynamics in a wild population (Ezenwa & Jolles 2011). Macroparasite infections can cause chronic stress and suppress host immune function, resulting in increased susceptibility to secondary microparasite infection (Graham 2008; Tort 2011). For example, laboratory experiments involving channel catfish, *Ictalurus punctatus* (Rafinesque) show that coinfection with another parasite can facilitate *Edwardsiella ictaluri* infection (Labrie, Komar, Terhune, Camus & Wise 2004; Xu, Shoemaker, Martins, Pridgeon & Klesius 2012; Shoemaker, Martins, Xu & Klesius 2012). Additionally, mechanical damage due to macroparasite attachment and penetration may also facilitate bacterial infection (Pylkkö, Suomalainen, Tiirola & Valtonen 2006; Bandilla, Valtonen, Suomalainen, Aphalo & Hakalahti 2006).

Many wild salmonid populations are listed as threatened under the Endangered Species Act in the USA Pacific Northwest (PNW) region (NMFS 2011). Given that disease may be a contributing factor to the modulation of population dynamics (Arkoosh, Clemons, Kagley, Stafford, Glass, Jacobson, Reno, Myers, Casillas, Loge, Johnson & Collier 2004; Ogut & Reno 2004), it is critical to understand how within-host interactions involving macro- and microparasites may affect disease in wild salmonid populations. Surveys document a high prevalence (60% - 100%) of an endemic macroparasite, the digenean trematode *Nanophyetus*

salmincola (Chapin) in wild juvenile Chinook, *Oncorhynchus tshawytscha*
(Walbaum) and coho *Oncorhynchus kisutch* (Walbaum) salmon, in various PNW
watersheds (Arkoosh *et al.* 2004; Ferguson, St-Hilaire, Peterson, Rodnick & Kent
2011). This trematode utilizes juvenile salmonids as a second intermediate host as
cercariae of *N. salmincola* penetrate and encyst as metacercariae in soft tissues of a
fish host, predominantly in the posterior kidney, where they remain encysted until the
fish is consumed by a definitive host, either a mammal or piscivorous bird
(Bennington & Pratt 1960). High numbers of *N. salmincola* cercariae can cause direct
mortality in salmon fry (Baldwin, Millemann & Knapp 1967), with pathogenicity
being related to the physical damage from cercariae penetration and migration rather
than the number of encysted metacercariae (Butler & Millemann 1971). However,
field and laboratory observation indicate that once encysted, *N. salmincola*
metacercariae can have negative effects on juvenile salmonid fitness and survival
parameters (Baldwin, Millemann & Knapp 1967; Foott, Free, Talo & Williamson
1997; Ferguson, Romer, Sifneos, Madsen, Schreck, Glynn & Kent 2012). Jacobson et
al. (2003) demonstrated that encysted *N. salmincola* metacercariae can reduce B cell
function and increase susceptibility to the marine bacteria, *Vibrio anguillarum*, with
potential negative effects on early marine survival (Jacobson, Teel, Van Doornik &
Casillas 2008). Out-migrating juvenile salmonids may accumulate a high *N.
salmincola* intensity (number of metacercariae/infected fish) throughout their
migration as the freshwater *Juga* spp. snail intermediate host is distributed throughout
PNW watersheds (Furnish 1990).

Consequently, juvenile salmonids that accumulate a high *N. salmincola* intensity may be at greater disease risk if coinfection increases susceptibility to microparasite infection. *Flavobacterium columnare* and *Aeromonas salmonicida* are ubiquitous in the freshwater environment (Austin & Austin 2012) and commonly linked to mortality in hatchery and wild salmonid populations in the PNW (Van Gaest, Dietrich, Thompson, Boylen, Strickland, Collier, Loge & Arkoosh 2011). Both of these bacteria are maintained in the environment through subclinical carrier fish or environmental reservoirs (Bullock & Stuckey 1975; Suomalainen, Tiirola & Valtonen 2005a; Kunttu, Sundberg, Pulkkinen & Valtonen 2012). Epizootics of *F. columnare* and *A. salmonicida* are more likely to occur during periods of increased temperature, low river flow, and high fish density (Austin & Austin 2012), which are environmental conditions that often coincide with migration of juvenile salmonids during late summer or early autumn (Friesen, Vile & Pribyl 2007). For example, peak migration of juvenile Chinook salmon in the Willamette River is from June to July, with some juvenile fish moving as late as November (Billman, Whitman, Schroeder, Sharpe, Noakes & Schreck 2014).

Here, we present results from a series of freshwater bacterial challenges designed to investigate the potential effects of *N. salmincola* infection on disease susceptibility. We hypothesize that encysted *N. salmincola* metacercariae will facilitate increased susceptibility to bacterial infection in juvenile Chinook salmon. Our primary goal is to extend beyond studies of single parasite infections and develop a greater understanding of coinfection in an aquatic environment, yielding insight into

the potential effects that parasite coinfection may have on the ecology of disease in wild salmonid populations.

Methods

Fish

Juvenile Spring Chinook salmon (~1.5g) were obtained from Marion Forks fish hatchery (Idanha, OR, USA) in June 2013. Fish were transported in an aerated tank to the John L. Fryer Salmon Disease Laboratory (SDL; Corvallis, OR, USA) where they were held in 379 L circular, aerated, flow-through tanks supplied with pathogen-free well water (13˚C) for 6 weeks prior to initiation of study. Unless otherwise noted, all experiments were done in aerated, flow-through tanks supplied with 13˚C water at 2 L min^{-1} flow. Fish were fed 1-2% body wt d^{-1} with appropriate sized fish feed (Bio-Oregon, Inc., Warrenton, OR, USA). Fish were not fed 24 hours prior to and after bacterial challenges. All procedures utilizing fish were approved by the Oregon State University (OSU) Institutional Animal Care and Use Committee.

Snails

Freshwater *Juga* spp. snails, an intermediate host of *N. salmincola*, were gathered from coastal rivers (Siletz, Alsea River), Willamette River tributaries (Luckiamute, McKenzie, South Santiam River), and the Willamette River main stem near the SDL. Collected snails were transported to the SDL and placed in single well of a 12-well tissue culture plate (1 snail/well). Wells were filled with 13˚C well water and a piece of organic lettuce, and snails were incubated at room temperature (21˚C) overnight. The next day, wells were examined for *N. salmincola* cercariae, the stage infective to fish. Snails shedding *N. salmincola* cercariae were maintained in 100 L

flow-through tanks for up to 8 weeks prior to fish exposure until sufficient numbers of *N. salmincola*-infected snails were collected for the cohabitation exposure.

Nanophyetus salmincola exposure

Exposures were conducted in 100 L tanks, in which fish were randomly assigned to either the control or *N. salmincola* treatment group. Treatments were replicated four times for a total of 8 tanks. Fish (n =125/tank) were cohabitated with *N. salmincola*-infected *Juga* spp. snails over a four month period. Control fish (n =125/tank) were held in identical tanks without snails. At the start of the cohabitation, there were 20 *N. salmincola*-infected snails/tank. To increase *N. salmincola* transmission, 10 infected snails were added weekly to each cohabitation tank until there were 60 snails/tank. Snails were allowed to freely roam the tank and were fed weekly with organic lettuce. Five fish were sub-sampled at 2, 6, and 10 week intervals to quantify progression of *N. salmincola* infections. Parasite intensity was quantified within the posterior kidney, as high concentrations of *N. salmincola* have a propensity to encyst in this tissue (Jacobson *et al.* 2008). To determine if fish had pre-existing *F. columnare* or *A. salmonicida* infections, gill and kidney tissue from the 20 sub-sampled fish were plated on TYES agar and TSA and incubated at 21˚C for 72 h. All sub-sampled fish were negative for *F. columnare* or *A. salmonicida* prior to bacterial challenges.

Bacteria and culture conditions

Isolates of *F. columnare* and *A. salmonicida* were previously lyophilized and kept at -20˚C until use. *F. columnare* was originally isolated from a gill lesion of a juvenile spring Chinook salmon with columnaris at Dexter Ponds Rearing Facility

(Lowell, OR, USA). *A. salmonicida* was originally isolated from the kidney of a juvenile winter steelhead, *Oncorhynchus mykiss* (Walbaum), with furunculosis at Rock Creek Hatchery (Idleyld Park, OR, USA). A stock of *F. columnare* was created by rehydrating the lyophilized bacteria with 2 mL of tryptone yeast extract salts broth (TYES; 0.4% tryptone, 0.04% yeast extract, 0.05% calcium chloride, 0.05% magnesium sulfate, pH 7.2; Cain & LaFrentz 2007) and incubating on TYES agar plates for 72 h at 21°C. Individual colonies were then removed and inoculated in 50 mL of tryptone yeast infusion broth (TYI; 0.4% tryptone, 3.0% yeast infusion, pH 7.2; Pacha & Ordal 1970) for 24 h at 21°C on a shaker set at 140 rpm. A 1 ml aliquot was mixed with 20% glycerol and maintained at -80°C until used for bacterial challenges. Prior to challenges, *F. columnare* was cultured as previously described. A 50 mL culture was used to inoculate 500 mL culture flasks with TYI broth. After 24 h, the OD_{525} was measured using a Spectronic 20 (Thermo Scientific) and was adjusted with sterile TYI broth to 1.0. *A. salmonicida* was cultured in the same manner, instead inoculating in tryptic soy broth (TSB; BD Biosciences) at 18°C on a shaker set at 200 rpm. We were unable to maintain a viable stock of *A. salmonicida* at -80°C. Therefore, rehydrated *A. salmonicida* colonies were inoculated and cultured as described above and used for a challenge to determine the dose required to induce 50% mortality (LD_{50}). *A. salmonicida* was re-isolated from kidney tissue of three fish that died during the LD_{50} challenge; these isolates were combined and cultured as previously described and used for the *A. salmonicida* coinfection experiment. Culture purity was confirmed by Gram stain and incubation on agar plates. *F. columnare* was inoculated on TYES agar plates and incubated for 72 h at 21°C. The isolate grew as

yellow, adhesive, rhizoid colonies that were motile, long, thin, Gram-negative rods. *A. salmonicida* was inoculated on tryptic soy agar plates (TSA) and incubated for 5 days at 18˚C. The bacteria produced a brown water-soluble pigment on TSA and were catalase and oxidase positive, nonmotile, Gram-negative rods. To determine colony forming units (cfu) mL^{-1} of challenge inoculum, 10-fold serial dilutions were performed and 50 µl was spread plated onto agar (TYES for *F. columnare*, TSA for *A. salmonicida*) plates (in triplicate). Plates were incubated for 72 h at 21˚C, and colonies were counted.

LD$_{50}$ determination

The LD$_{50}$ for *F. columnare* was based on Holt et al. (1975), who cultured *F. columnare* for 24 h and diluted to a 0.1 OD$_{525}$ (10^6 cfu mL^{-1}). Prior to challenge, the water temperature was gradually increased from 13˚C to 18˚C over a few hours. We challenged duplicate groups of 10 fish in 25 L tanks with dilutions of a *F. columnare* culture at 0.1, 0.15, and 0.25 OD$_{525}$ (1.5 X 10^6, 2.0 X 10^6, and 4.5 X 10^6 cfu mL^{-1}, respectively) using a 1 h static immersion challenge at 18˚C. The LD$_{50}$ for this *F. columnare* isolate corresponded to the 0.15 OD$_{525}$ and was achieved by diluting 40 mL of 1.0 OD$_{525}$ culture in 500 mL of tank water before adding directly to the tank at a 1:20 dilution (v/v). The LD$_{50}$ for *A. salmonicida* was determined by challenging duplicate groups of 8 fish in 25 L tanks to low (3.6 X 10^6 cfu mL^{-1}) and high (2.9 X 10^7 cfu mL^{-1}) concentrations using a 30 min static immersion challenge at 18˚C. The LD$_{50}$ for this *A. salmonicida* isolate corresponded to the low concentration and was achieved by diluting 30 mL of 1.0 OD$_{525}$ culture in 500 mL of tank water before adding directly to the tank at a 1:20 dilution (v/v).

Experiment 1: F. columnare challenge

After *N. salmincola* exposure, fish (46.9g ± 1.2) were randomly assigned to 25 L tanks (*n* = 10 fish/tank) with a 2 X 2 factorial design, in which *N. salmincola*-parasitized or control fish were challenged with either *F. columnare* or sterile TYI broth. Treatments were replicated three times for a total of 12 tanks. Treatments were designated as control (C), parasitized by *N. salmincola* (N), challenged with *F. columnare* (Fc), and parasitized by *N. salmincola* and challenged with *F. columnare* (N+Fc; coinfection). After transfer, the water temperature was gradually increased from 13°C to 18°C over a few hours. Fish were acclimated to this temperature for 3 days prior to *F. columnare* challenge. Fish in the Fc and N+Fc treatments were challenged with 1.96×10^6 cfu mL^{-1} of *F. columnare* with interrupted water flow for 60 min by adding a 1:20 dilution (v/v) of a 1.0 OD$_{525}$ (9.8×10^8cfu mL^{-1}) culture to each tank. Fish in the C and N treatments were challenged in the same manner with diluted, sterile TYI broth.

Experiment 2: A. salmonicida challenge

Control and *N. salmincola*-parasitized fish (41.9g ± 2.0) used for a separate pilot study and only exposed to sterile TYI broth were used for the *A. salmonicida* challenge. These fish were held for a similar time after the initial snail cohabitation period (reared 8 months total) as the fish used in Experiment 1, but in 25 L tanks. These were then randomly assigned to new 25 L tanks (*n* = 10 fish/tank) in the same 2 X 2 factorial design as described above. Treatments were designated as control (C), parasitized by *N. salmincola* (N), challenged with *A. salmonicida* (As), and parasitized by *N. salmincola* and challenged with *A. salmonicida* (N+As;

coinfection). Fish were acclimated at 13˚C for 3 days prior to *A. salmonicida*
challenge, and upon resumption of the water flow following challenge, the inflow was
supplied at 18˚C. The As and N+As treatments were challenged with 1.20×10^6 cfu
mL^{-1} of *A. salmonicida* with interrupted water flow for 30 min by adding a 1:20
dilution (v/v) of a 1.0 OD_{525} (8.0×10^8cfu mL^{-1}) culture to each tank. The C and N
treatments were challenged in the same manner with diluted, sterile TSB.

Fish monitoring

During all disease challenges, fish were monitored twice daily for clinical
disease signs, such as lesions, darkening, lethargy, loss of equilibrium, and lack of
feeding. Mortalities were removed every 12 hours and moribund fish or fish surviving
at the termination of an experiment were euthanized with 250 mg L^{-1} of buffered
tricaine methanesulfonate (MS-222, Argent Chemical Laboratories). Clinical signs
were recorded along with weight (g) and fork length (mm) for evaluation of condition
factor (weight/length^3). Kidneys were cultured on TSA and TYES agar plates for
bacterial growth. Posterior kidneys from each fish were collected in Stomacher® bags
(Seward Ltd.) and frozen at -20˚C for later quantification of *N. salmincola*
metacercariae.

Necropsy and diagnostic confirmation

For *F. columnare* challenged fish, gill and mid-kidney tissue were streaked
onto separate TYES agar plates. Plates were incubated at 21˚C and examined at 3 and
7 d for bacterial growth. Presumptive identification for *F. columnare* was observation
of yellowish lesions and necrosis on the gills and body of the fish, presence of
haystacks/columnar structures on gill through wet mount examination, and growth of

rhizoid, adhesive, yellow colonies. For *A. salmonicida* challenged fish, the mid-kidney was streaked onto TSA plates. Presumptive identification of *A. salmonicida* was observation of furuncle lesions on the body of the fish and growth of colonies causing brown pigment growth on TSA. *N. salmincola* metacercariae were quantified by defrosting and weighing the posterior kidney tissue subsamples. Tissue was then squashed within the Stomacher bag using a glass slide and examined using a binocular dissection microscope at $50 - 100\times$ magnification (Ferguson *et al.* 2010). Mean intensity was defined as the average number of *N. salmincola* metacercariae per posterior kidney among all the infected fish.

Statistical analysis

Logistic regression models with a random intercept for tank were used to fit the cumulative percent mortality data from each challenge. This model assumes that mortality and survivor data follow a binomial distribution (Jacobson, Arkoosh, Kagley, Clemons, Collier & Casillas 2003). A likelihood ratio test was used to evaluate if coinfection mortality differed from the baseline mortality from bacteria alone. Mean values were considered significantly different at $P < 0.05$. Mean *N. salmincola* intensity was compared between survivors and mortalities within coinfection treatments (N+Fc, N+As) using a Welch's two-sample *t*-test. Cumulative percent mortality results are reported as mean $\pm$ 1 SD, otherwise results are reported as mean $\pm$ 1 SE.

Results

For the *F. columnare* coinfection challenge, the N+Fc treatment had significantly higher mean cumulative percent mortality (80.0% ± 0.0%) than the Fc treatment (50.0% ± 10.0%) at the conclusion of the experiment (X^2 = 5.63, P = 0.018). This *F. columnare* isolate caused acute mortality, with the majority of deaths occurring 1-2 days post challenge (dpc; Fig. 1). *F. columnare* was isolated from the gills of all disease-related mortalities (n =39) during the experimental period, and isolated from the kidney of one fish that died 5 dpc. External clinical disease signs were largely absent, but yellow gill lesions were observed in a few mortalities (8%). *F. columnare* was not detected in the gills or kidney tissue from any surviving fish at 10 dpc. No disease-related mortality was observed in the C or N treatments. Prior to bacterial challenge, all *N. salmincola*-parasitized fish had metacercariae encysted in the posterior kidney (range, 111-526; mean intensity, 245.5 ± 10.0). Overall, there were no significant differences between mean *N. salmincola* intensity among and between treatments containing *N. salmincola*-parasitized fish ($F_{11, 109}$ =1.126, P = 0.348). At the individual level, mean *N. salmincola* intensity did not significantly (Welch's two sample *t*-test, t = -1.12, d.f. = 7.43, P = 0.300) differ between survivors (293.67 ± 0.37) and mortalities (239.67 ± 0.17) within the coinfection treatment. There were no significant differences in mean weight, length, or condition factor between treatment groups in this challenge.

For the *A. salmonicida* coinfection challenge, mean CPM in the N+As treatment (53.3% ± 14.5%) was marginally higher than observed in the As treatment (46.7% ± 13.3%). Although mortality was delayed in the As treatment, it was not

significantly ($X^2 = 0.18$, $P = 0.674$) different at the conclusion of the experiment.

Mortality from this *A. salmonicida* isolate occurred from 4-21 dpc (Fig. 2). *A. salmonicida* was isolated from the kidneys of all disease-related mortalities during the experimental period. External clinical disease signs (furuncle lesions) were observed in all disease-related mortalities ($n = 30$). *A. salmonicida* was not detected in the kidneys of any surviving fish at 30 dpc. No disease-related mortality was observed in the C or N treatments. Prior to bacterial challenge, all *N. salmincola*-parasitized fish had metacercariae encysted in the posterior kidney (range, 82-439; mean intensity, 239.2 ± 13.2). Overall, there were no significant differences between mean *N. salmincola* intensity among and between treatments containing *N. salmincola*-parasitized fish ($F_{5, 54} = 1.366$, $P = 0.252$). There was a marginally significant (Welch's two sample *t*-test, $t = -1.76$, d.f. $= 23.14$, $P = 0.092$) difference between mean *N. salmincola* intensity in survivors (244.71 ± 0.19) and mortalities (190.19 ± 0.23) within the coinfection treatment. There were no significant differences in mean weight, length, or condition factor between treatment groups in this challenge.

N. *salmincola* intensity was not associated with fish length, weight, or condition factor (data not shown) in either challenge. Mean *N. salmincola* intensity in the *F. columnare* coinfection tanks (249.67 ± 19.20) was not significantly ($F_{5, 54} = 1.145$, $P = 0.348$) different from the *A. salmonicida* coinfection tanks (215.63 ± 15.69).

Discussion

Using a laboratory challenge method we showed that encysted *N. salmincola* can affect disease susceptibility of juvenile Chinook salmon, but the extent of

coinfection effects may depend on the challenging pathogen. Our results support previous studies that have shown that encysted *N. salmincola* metacercariae increase susceptibility of juvenile Chinook salmon to the marine bacteria, *Vibrio anguillarum* (Jacobson *et al.* 2003). Taken together, these findings suggest that *N. salmincola* can affect susceptibility to secondary microparasites, which may affect the ecology of disease in wild Chinook salmon populations.

Wild juvenile Chinook salmon rearing in tributaries and migrating during late summer or early autumn may accumulate high *N. salmincola* intensities, as infected *Juga silicula* snails have been shown to release *N. salmincola* cercariae starting at 10°C, with *N. salmincola* prevalence and cercariae release increasing with temperature (Gebhardt 1966) and are distributed throughout the tributaries and the Willamette River main stem. Wild juvenile Chinook salmon display a diverse range of migratory tactics in the Willamette River (Billman *et al.* 2014), but generally, spend time in-river during summer months and encounter areas of congestion while bypassing dams along the Willamette and Columbia River (Friesen *et al.* 2007). This may coincide with a period when conditions are favorable to epizootics of *F. columnare* or *A. salmonicida* (high temperature and low flow). Therefore, the challenge temperature (18°C) in this study is typical of temperatures recorded in the Willamette and Columbia River main stem during late summer or early autumn (Billman *et al.* 2014).

It is likely that *N. salmincola* coinfection effects a proportion of individuals within wild juvenile Chinook populations, as macroparasite intensities typically follow a negative binomial distribution in the wild, with few to no parasites in the

majority of hosts and high parasite intensities in a small percentage of hosts (Lester 1984; Ferguson *et al.* 2011). Given that higher mortality during the *F. columnare* challenge was associated with a high mean *N. salmincola* intensity within each tank, a threshold *N. salmincola* intensity (e.g. ~200 metacercariae) may be necessary to have any observable effect of coinfection on mortality within a juvenile Chinook population. We hypothesized that individuals with higher *N. salmincola* intensities would be more likely to succumb to bacterial infection. However, within coinfection treatments from both challenges, a trend of lower mean *N. salmincola* intensities in mortalities compared to survivors did not support this hypothesis. The explanation for this is not clear. Given that some hosts can tolerate parasite effects, it is possible that surviving fish had an increased immune competence or ability to tolerate effects of *N. salmincola* (Råberg, Graham & Read 2009).

Differences in virulence and horizontal transmission may explain the contrast in effects of *N. salmincola* coinfection between the two challenges. Epizootics of these bacteria can progress at different timescales and rates of horizontal transmission (Ogut & Reno 2004; Suomalainen, Tiirola & Valtonen 2005b). In this study, the *F. columnare* challenge caused acute mortality within 2 days. In contrast, mortality following the *A. salmonicida* challenge progressed over a longer period (4-21 dpc), likely as a result of horizontal transmission. Furthermore, the difference between the two bacteria challenges may be attributed to dissimilarities in pathogenesis between the two bacteria. *F. columnare* infections are typically external, associated with attachment and erosion of the gills and skin (Decostere, Haesebrouck, Van Driessche,

Charlier & Ducatelle 1999), whereas *A. salmonicida* can affect many internal organs, presenting disease as an internal hemorrhagic septicemia (Austin & Austin 2012).

These results agree, in part, with previous studies that have shown *N. salmincola* can increase susceptibility of juvenile Chinook salmon to *V. anguillarum* (Jacobson *et al.* 2003). Though *A. salmonicida* and *V. anguillarum* both cause systemic, hemorrhagic septicemia in salmonids, Jacobson *et al.* (2003) challenged smaller fish (9.2g) with a higher mean *N. salmincola* intensity (394; range, 257-504) in saltwater. Therefore, it is difficult to compare the effects of coinfection as saltwater acclimatization (smoltification) is a drastic physiological shift for salmonids (Maule, Tripp, Kaattari & Schreck 1989) and may have altered the effect *N. salmincola* had on the host.

N. salmincola metacercariae can decrease B cell function in parasitized juvenile Chinook, so it has been hypothesized that immunosuppression from encysted *N. salmincola* may increase disease susceptibility (Jacobson *et al.* 2003). Histopathology of *N. salmincola* infection indicates mild, chronic inflammation around encysted metacercariae (Ferguson *et al.* 2010). This may lead to suppressive effects on immune response as a result of chronic stress (Salonius & Iwama 1993; Tort 2011) and to deterioration of host condition as an immune response consumes energetic resources (Barber & Wright 2005). Therefore, an indirect, within-host interaction caused by a suppressed immune response may explain why *N. salmincola*-parasitized fish were more susceptible to *F. columnare*. However, given the evidence for immunosuppressive effects of *N. salmincola* it was unexpected that a general effect of coinfection on mortality was not observed in both challenges. Therefore, the

mechanism underlying a within-host interaction may be complex, as fish immune systems react differently to different parasite types (Whyte 2007).

In conclusion, we have demonstrated that encysted *N. salmincola* metacercariae can significantly affect susceptibility of juvenile Chinook salmon to the bacterium *F. columnare*. Infections of *N. salmincola* metacercariae and bacterial infections may lead to higher mortalities in coinfected Chinook salmon populations. However, it appears that encysted *N. salmincola* metacercariae effects are more complex than hypothesized, given that a coinfection effect on mortality was not observed in both bacterial challenges. Further investigation of immune responses to *N. salmincola* and coinfection within fish is needed to explain if a within-host interaction exists and will help predict other macro- and microparasite coinfection outcomes in Chinook salmon. Additional experiments at lower temperatures and/or lower *N. salmincola* intensities would reveal if coinfection presents a heightened risk to juvenile Chinook salmon outside our experimental conditions. Finally, incorporating coinfection into epidemiological models may elucidate how *N. salmincola* coinfection influences disease dynamics in wild salmonids.

Acknowledgements

in maintaining and monitoring fish. We gratefully acknowledge Amy Long and Steve

Whitlock for their critical review of this manuscript and statistical assistance.

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Table 3.1. Cumulative percent mortality of juvenile Chinook salmon as a result of *F. columnare* or *A. salmonicida* challenge of *N. salmincola*-parasitized fish and non-parasitized (control) fish. Treatments without bacteria of *N. salmincola*-parasitized fish and non-parasitized (control) fish did not show any mortality. The values are the number of mortalities/the total number; percentages of mortalities are in parentheses. The mean cumulative percent mortality is shown ± 1 SD.

	Flavobacterium columnare		*Aeromonas salmonicida*	
	Control	*N. salmincola*	Control	*N. salmincola*
Replicate				
1	4/10 (40%)	8/10 (80%)	6/10 (60%)	5/10 (50%)
2	4/10 (40%)	8/10 (80%)	6/10 (60%)	3/10 (30%)
3	7/10 (70%)	8/10 (80%)	2/10 (20%)	8/10 (80%)
Mean	50.0% ± 10.0	80.0% ± 0.0*	46.7% ± 13.3	53.3% ± 14.5

* Indicates significant difference from control group ($P < 0.05$)
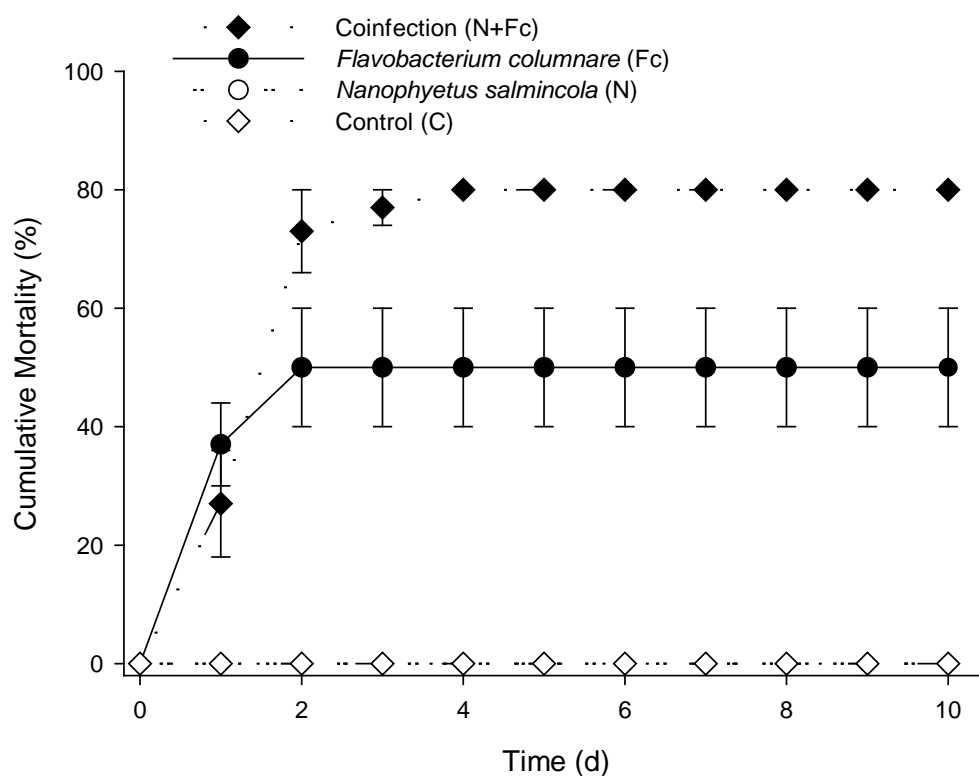
Figure 3.1. Cumulative percent mortality (mean ± SD) of juvenile Chinook salmon *Oncorhynchus tshawytscha* in one of four treatments in Experiment 1: encysted *Nanophyetus salmincola* metacercariae challenged with *F. columnare* (N+Fc), *F. columnare* (Fc) only, encysted *N. salmincola* (N) only, and a negative control. The difference in cumulative percent mortality between the coinfection and Fc-only groups on day 10 were significant (*P*= 0.018).
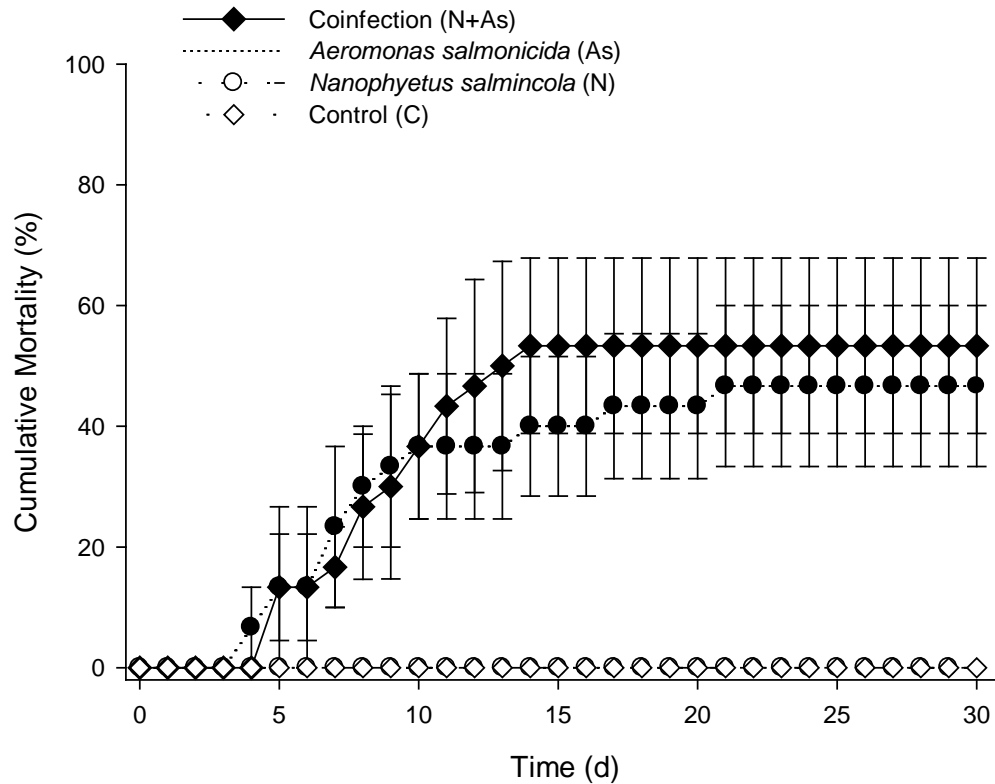
Figure 3.2. Cumulative percent mortality (mean ± SD) of juvenile Chinook salmon *O. tshawytscha* in one of four treatments in Experiment 2: encysted *Nanophyetus salmincola* metacercariae challenged with *A. salmonicida* (N+As), *A. salmonicida* (As) only, encysted *N. salmincola* (N) only, and a negative control. The difference in cumulative percent mortality between the coinfection and As-only groups at day 30 was not significant.

CHAPTER 4: SUMMARY

Chapter 2: Micro- and macroparasite survey of wild juvenile Chinook salmon and steelhead/rainbow trout from three upper Willamette tributaries.

- Select microparasites: *Aeromonas salmonicida*, *Flavobacterium columnare*, *Flavobacterium psychrophilum*, and IHNV were not detected in wild juvenile Chinook salmon and rainbow trout populations.

- There was a low prevalence of *Renibacterium salmoninarum* in both salmonid species collected from the three sites, but there were no spatial differences in prevalence.

- In 2013, prevalence of *R. salmoninarum* in Chinook salmon collected at the MCK site significantly decreased between the summer and autumn sampling periods.

- Despite no detection of *F. columnare* in wild salmonids, isolation from resident sculpin indicates a presence of *F. columnare* in this upper Willamette River and that non-salmonid species may be a reservoir.

- 23 metazoan parasite species were identified, none of which are generally linked to high mortality events in salmonids. *Nanophyetus salmincola* was the most common parasite.

- In 2012, steelhead/rainbow trout parasite richness was significantly lower at the MFW site compared to parasite richness at SS and MCK. Ordinations of parasite communities reflected a nested structure with low parasite richness associated with the presence of species of trematode metacercariae and high

parasite richness associated with the additional presence of species of myxozoans, adult trematodes, nematodes, and cestodes.

- NMDS reflected a negative association between parasite richness and temperature.

Chapter 3: Coinfection effects of *Nanophyetus salmincola* (Chapin) and two pathogenic bacteria on Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum)

- Encysted *N. salmincola* made Chinook salmon more susceptible to *F. columnare*-induced mortality, with a difference of ~30% in cumulative percent mortality between bacteria and coinfection groups.

- Cumulative percent mortality from *A. salmonicida* did not differ between *N. salmincola*-parasitized and non-parasitized groups.

- Encysted *N. salmincola* metacercariae interactions with bacteria are more complex than hypothesized, given that a coinfection effect on mortality was not observed in both bacterial challenges.

- Differences in onset of mortality may be related to virulence and horizontal transmission rates. *F. columnare* primarily infected the gills causing acute mortality within 2 days. In contrast, mortality following the *A. salmonicida* challenge progressed over a longer period (4-21 dpc), which may be due horizontal transmission.

SYNTHESIS OF PARASITE DISTRIBUTION AND COINFECTION

The important role of parasitic communities in diversity-disease interactions and the ubiquity of metazoan parasites across wild salmonid populations creates a critical need to establish information regarding within-host parasite interactions as these can

be a factor driving disease prevalence and transmission patterns (Telfer *et al.* 2010; Ezenwa & Jolles 2011).

- Due to low detection of selected microparasites, coinfection between micro- and macroparasites was not commonly observed in the upper Willamette tributaries.

- However, when *R. salmoninarum* was detected, the individual fish was commonly coinfected with another parasite. Overall, 82% of *R. salmoninarum*-positive Chinook salmon ($n = 33$) were coinfected with *N. salmincola*. This particular pathogen was not investigated as culturing this bacteria, and subsequent chronic mortality, can take months. Therefore, this created an inherent time constraint and presented a timeline not feasible for this current thesis.

- The occurrence of *F. columnare* epizootics at the Dexter Ponds rearing facility indicate the bacteria is present in close proximity (Pearsons *et al.* 2012), and the presence of different parasites could affect disease distributions in the upper Willamette River.

- Characterizing how parasite interactions may affect disease susceptibility of out-migrating juvenile Chinook salmon, whether hatchery or wild, is critical to elucidate underlying drivers of disease.

FURTHER DISCUSSION OF WITHIN-HOST IMMUNE RESPONSE REGARDING COINFECTION

Confined selection of antibodies and slow proliferation, low maturation rates, weak affinity, and limited memory of lymphocytes all restrict the adaptive response in fish (Tort, Balasch & Mackenzie 2003; Uribe *et al.* 2011). Therefore, the fish's innate response is considered the primary component in fighting infection (Whyte 2007). A variety of factors can trigger an innate response, which may be stimulated, suppressed, or escaped in various ways, by various parasites (Ellis 2001; Sitjà-Bobadilla 2008).

- Differences in mortality and disease progression between the two challenges may indicate that *N. salmincola* has a negative effect on the acute phase of the innate response, but its effects may be less pronounced if an infection takes longer to progress.

- Chronic inflammation around *N. salmincola* metacercariae may suppress innate responses important for fighting off specific bacteria. Suppression of external gill responses may have decreased the ability to resist *F. columnare* attachment resulting in the increased coinfection mortality. Whereas, internal immune responses critical for controlling *A. salmonicida* may not have been suppressed.

- For example, the immunostimulant β-1,3 glucan stimulates plasma lysozyme activity, plasma complement bacteriolytic activity, and reactive oxygen species (ROS) production by blood and head kidney leukocytes and has been demonstrated to increase resistance of Chinook salmon and brook trout

against *A. salmonicida* (Nikl, Evelyn & Albright 1993; Anderson & Siwicki 1994). However, these immunostimulants did not increase resistance of rainbow trout to *F. columnare* infection (Kunttu *et al.* 2009b).

- Tissue damage from *F. columnare* infection primarily occurs externally and virulence is associated with gill attachment (Kunttu *et al.* 2009a), which may indicate that mucosal antibody is an important inhibitor of this bacteria (Grabowski, LaPatra & Cain 2004).

  o Rhamnose-binding lectin, a protein that binds to surface carbohydrates of bacteria, is stimulated in catfish gills challenged with *F. columnare* (Sun *et al.* 2012).

  o In zebrafish (*Danio rerio*), RNAi-mediated suppression of the peptidoglycan recognition protein, zfPGRP6, important for the regulation of Toll-like receptors resulted in increased susceptibility to *F. columnare* (Chang & Nie 2008).

- Due to similar disease and pathogenesis of *A. salmonicida* and *Vibrio anguillarum* to salmonids, it seems likely these bacteria would have similar results when coinfected with *N. salmincola.* However, there was not a significant effect on cumulative percent mortality in the current study involving *A. salmonicida,* while Jacobson et al. (2003) completed coinfection challenges resulting in a significant difference in cumulative percent mortality between *V. anguillarum* (43%) and *V. anguillarum/N. salmincola* coinfection groups (75%). This may be explained by differences in challenge variables:

- o Jacobson et al. (2003) used smaller fish (9.2g) with a higher mean *N. salmincola* intensity (394; range, 257-504).

- o The *V. anguillarum* challenge was done in saltwater, therefore, requiring a change in osmoregulation by the juvenile Chinook salmon.

FUTURE RESEARCH

Transmission between hatchery and wild fish

The extent of transmission between hatchery and wild fish still remains unknown

(Rand *et al.* 2012). It was an objective of this survey to provide baseline information

on microparasite and macroparasite distributions so that further research into

pathogen transmission between these fish could be assessed. However, with little

detection of select microparasites, no conclusions can be made regarding disease

transmission or any effects on hatchery and wild fish.

- From 2011-2013, Dexter Ponds Rearing Facility experienced annual high mortality events of their population of juvenile salmonids, due to either *Flavobacterium columnare* or *Aeromonas salmonicida*. *F. columnare*, is of special concern because it has consistently caused epizootics at Dexter Ponds over the last 25 years, sometimes resulting in losses of 60-90% (Amandi 1997). It is typically a problem during the late summer and fall, when water temperatures can rise to about $18^{\circ}C$ (Wakabayashi 1991). In 2012 and 2013, wild salmonids collected downstream from the facility undergoing *F. columnare* and *A. salmonicida* epizootics but neither bacteria were not detected. This may be due to dilution of any microparasite in the effluent, reducing the transmission rate to susceptible individuals. Epizootics in wild

fish are difficult to document and may be missed during sampling, because dead or moribund fish tend to be quickly taken out of the system by natural events such as predation (Sindermann 1987; Hedrick 1998; Jacobson *et al.* 2008).

- *F. columnare* was isolated from resident sculpin providing evidence that the bacteria was present in the river near the facility.

- Further efforts will benefit from sampling with increased knowledge of the likelihood of detecting these specific microparasites, and future surveys can focus on establishing seasonal prevalence, which may be related to coinciding epizootic dynamics in nearby hatchery facilities giving a more concise connection between wild and hatchery populations. This would require more frequent sampling efforts at structured time intervals.

- Subsequent surveys should utilize more resolute measures of age (otolith or scaling) to evaluate if differences in age or out-migration strategies relate to parasite prevalence, intensity, distribution, or infracommunity composition. For example, previous research near the MCK site, has revealed complex outmigration strategies, with some juveniles immediately moving down river, while others stay in the McKenzie River for up to 2 years (Billman *et al.*, 2014)

Given that natural communities are composed of a diverse assemblage of parasitic and nonparasitic microorganisms, there is a growing need to explore the outcome of interactions among parasite species across a range of systems and conditions (Johnson & Hoverman 2012). Studying coinfection interactions and further

characterization of fish immune response to macroparasites, especially to digenean

metacercariae is needed to elucidate interaction outcomes with different

microparasites. This may reveal general interaction trends of coinfection outcomes in

fish based on the community of parasites present in a given system.

- Interactions with other digenean trematodes (*Apophallus* sp.) might have a

  similar impact on disease susceptibility, therefore, it is critical to understand

  general within-host response to these macroparasites.

- Knowledge of local gill and systemic immune responses would help predict

  interactions between microparasites. There may be organ specific transcriptive

  immune responses to *N. salmincola* metacercariae, as shown by Pacific

  bluefin tuna *Thunnus orientalis* to blood flukes (Polinski *et al.* 2014).

- Establish coinfection effects of *N. salmincola* and *R. salmoninarum*, as this

  may be a common parasite interaction within wild salmonids.

How may macro- and microparasite coinfections affect population dynamics?

- Any action undertaken to mitigate a single disease could have unintended

  consequences for a system where coinfection is common. Simple

  epidemiological models have been successfully used to predict infection

  patterns of *Mycobacterium bovis* in African buffalo (Jolles *et al.* 2008).

  Adaptation of this model could output predicted values of disease prevalence

  that could be used to generate and test hypotheses for coinfection effects on

  wild fish populations in conjunction with surveillance.

- Alteration to disease parameters by coinfection may significantly affect the

  individual, but not significantly affect population disease dynamics (Ezenwa

& Jolles 2011). A sharp increase in mortality may actually not have a population level effect as transmission efficiency would presumably decrease if diseased individuals quickly die and transmittion is curtailed.

- Repeated coinfection challenges with lower *N. salmincola* intensity (~25–50 metacercariae/posterior kidney) will reveal if an effect is possible without high *N. salmincola* intensity.

- Adapt future challenges to investigate how *N. salmincola* may affect susceptibility in other salmonid species, as well as other Chinook salmon stocks.

How might rearing in a habitat with more parasite diversity affect disease susceptibility?

- For example, habitats with greater macroparasite diversity have been correlated with increased immune response in threespine sticklebacks *Gasterosteus aculeatus* (Scharsack *et al.* 2007) and acute stress can actually boost an immune response (Martin 2009). Therefore, it is possible an active response to parasites in a natural system may lead to an indirect, competitive interaction with secondary infections through cross-immunity.

- Therefore, similar research with salmonids will reveal if fish reared with higher parasite diversity are more resistant to disease compared to salmonids if the immune response is stimulated by rearing with higher parasite diversity.

As the importance of parasite interactions are determined, traditional disease management strategies will benefit from community-based approaches.

- Wildlife disease management has largely focused on reducing density-dependent transmission, however, other concepts such as biodiversity or within-host parasite interactions have not been integrated to management efforts of wildlife (Joseph *et al.* 2013), as well as wild salmonids in the PNW.

- Control of the snail intermediate host is a recommended management strategy for coinfection issues involving digenean trematodes (Labrie et al. 2004). However, this method is not advisable in a wild system due to the potential for widespread, deleterious effects on the habitat.

- Consequently, research on how cercariae transmission rates are affected by more diverse fish communities will reveal if increased biodiversity decreases *N. salmincola* intensities within individual fish. Metacercariae of *N. salmincola* have been reported in a diverse array of hosts, including the Pacific giant salamander, lamprey, sculpin, and redside shiner (Gebhardt et al. 1966). Therefore, increased host biodiversity might alleviate high parasite intensities through a dilution effect, acting as biological "sponges" for cercariae.

- Focused studies on riparian habitat restoration and flow effects on disease may be conducted using mesocosm experimental design to simulate a natural system. This will help understand connections between ecosystem health and fish health.

Warming temperatures will affect disease ecology of salmonid populations, especially with regards to coinfection as the *Juga* spp. snail and free-swimming cercariae will also be affected by temperature.

- Cercariae are produced at a higher rate with increasing temperatures, but other systems have shown a temperature threshold can be reached, causing a decrease in transmission (Paull, LaFonte & Johnson 2012).

- Warming temperatures can also negatively affect snail host survival, which can significantly decrease parasite infection within an ecosystem (Paull & Johnson 2014).

- *F. columnare* transmission from carrier fish are not a risk if water temperature is kept low (Suomalainen, Tiirola & Valtonen 2005).

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APPENDICES

APPENDIX A: CERCARIAL PENETRATION OF *NANOPHYETUS SALMINCOLA* AS A POSSIBLE MECHANICAL PATHWAY FOR INCREASED SUSCEPTIBILITY TO *FLAVOBACTERIUM COLUMNARE*.

Introduction

A growing body of literature documents that an established macroparasite can facilitate or enhance a microparasite infection within a fish host (Xu *et al.* 2012; Shoemaker *et al.* 2012). This interaction may arise from the macroparasite adding chronic stress and suppressing immune function, decreasing the ability of the host to fight off a secondary microparasite infection (Graham 2008; Tort 2011). Alternatively, mechanical damage from macroparasite attachment and penetration can also facilitate bacterial infection, absent of chronic effects from an established macroparasite infection (Labrie *et al.* 2004; Pylkkö *et al.* 2006; Bandilla *et al.* 2006).

Results from a previous co-infection challenge demonstrated that Chinook salmon with encysted *Nanophyetus salmincola* metacercariae were more susceptible to *Flavobacterium columnare* (Roon, Alexander, Jacobson, & Bartholomew 2014). In a natural system, there may be variable times of cercariae release and active penetration of *N. salmincola* cercariae may also be a possible pathway for facilitating bacterial infection if simultaneously exposed. I conducted a laboratory experiment to investigate if *N. salmincola* cercariae penetration alone can facilitate higher mortality from *F. columnare*.

Methods

Fish

Spring Chinook salmon (~1.5g) were obtained from Marion Forks fish hatchery (Idanha, OR, USA) in June 2013. These were from the same stock of fish as described in Chapter 3, they were maintained and treated as described above.

Snails

Freshwater *Juga* spp. snails were from the same stock as used in Chapter 3; these were maintained in 100 L flow-through tanks.

Bacteria and culture conditions

The *F. columnare* isolate was the same as described above in Chapter 3. An aliquot was mixed with 20% glycerol and maintained at -80˚C until used for bacteria challenges. All culture conditions and media for *F. columnare* were the same as described previously in Chapter 3.

F. columnare challenge

Fish (46.98g ± 1.2; mean weight ± SE) were randomly assigned to 25 L tanks (n = 10 fish/tank), in which control fish were challenged with either a dose of *N. salmincola* cercariae or well water, concurrently with either *F. columnare* or sterile TYI broth exposure. Treatments were replicated three times for a total of 12 tanks. Treatments were designated as control (C), exposed to *N. salmincola* cercariae (C+cercariae), *F. columnare* (Fc), and simultaneously challenged with *F. columnare* and *N. salmincola* cercariae (Fc+cercariae). Fish were challenged with *F. columnare* in the same manner as previously described in Chapter 3, but the Fc+cercariae group was simultaneously exposed to *N. salmincola* cercariae (747 ± 49). Fish in the C and

N treatments were challenged in the same manner with diluted, sterile TYI broth and/or *N. salmincola* cercariae (747 ± 49).

Fish monitoring

Fish monitoring, necropsy and tissue sampling, bacteria isolation, and *N. salmincola* metacercariae quantification was performed as described in Chapter 3.

Statistical analysis

Logistic regression models with a random intercept for tank were used to fit the cumulative percent mortality data from each challenge. A likelihood ratio test was used to evaluate if Fc + cercariae mortality differed from the baseline Fc mortality. Mean values were considered significantly different at $P < 0.05$. Cumulative percent mortality results are reported as mean ± 1 SD, otherwise results are reported as mean ± 1 SE.

Results

Simultaneous exposure to *N. salmincola* cercariae and *F. columnare* did not significantly ($X^2 = 0.07$, $P = 0.796$) affect *F. columnare*-induced mortality (Fig. A.1). Low *N. salmincola* intensities (7.1 ± 6.2) observed in the fish from the Fc+cercariae group indicate a low success rate of transmission (0.95% ± 0.83% of total cercariae dosage) during the immersion exposure. No disease-related mortality was observed in C or N treatment groups (Fig. A.1). *F. columnare* was re-isolated from the all of the mortalities (n=29) and not from any survivors at 10 days post challenge (dpc).

Discussion

No significant difference between mean cumulative mortality of the Fc group and Fc + cercariae group does not indicate that mechanical damage is a mechanism

for increasing *F. columnare* susceptibility in juvenile Chinook salmon. However, *N. salmincola* transmission was low or absent in many fish exposed to cercariae in either N or Fc+cercariae treatment, which would not initiate a large host response. Consequently, this does not provide conclusive evidence that cercariae penetration affects *F. columnare* susceptibility.

If cercarial penetration were to enhance bacterial infection, it could be expected it would increase *F. columnare*-induced mortality given that abrasion has been shown to increase Fc infection (Bandilla *et al*. 2006) Therefore, cercariae may have some affect breaking the mucosal barrier, but large numbers may need to penetrate to have an effect. Labrie (2004) observed increased cumulative percent mortality of catfish simultaneously exposed to the digenean trematode *Bolbophorus* spp. and *Edwardsiella ictaluri*, but did not observe any difference in mortality between catfish exposed to the trematode 28 d prior and then exposed to *E. ictaluri*. Cercarial penetration may not affect mortality, but may lead to increased bacterial invasion, presumably through mechanical damage from penetration. Pylkko et al. (2006) exhibited that *Diplostomum spathaceum* cercariae penetration did not affect mortality to *A. hydrophila*, but did increase bacteria load in fish that were exposed to cercariae. However, bacterial load was not quantified in the present study.

Cercariae observed during screening did not show self-generated propulsion into the water column, therefore, it is likely they are passively transmitted through water movement. The water volume used during the static immersion exposure may have been too large, decreasing the contact rate between cercariae and fish by limiting the contact area to the bottom of the tank. Finally, active cercariae penetration may

stimulate local immune response that may offer some cross-protection against *F. columnare*. Despite low success in establishing *N. salmincola* transmission, a slight decrease in mortality was observed in the Fc+cercariae group.
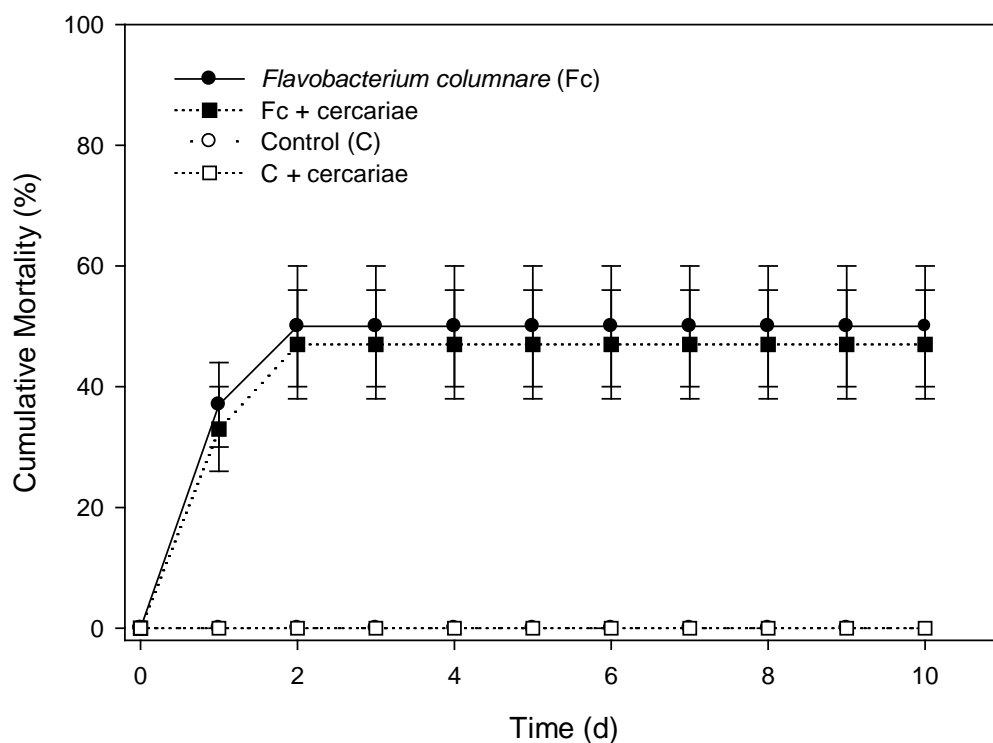
Figure A.1. Cumulative percent mortality (mean ± SD) experienced by juvenile Chinook salmon in one of four treatments 10 d post *Flavobacterium columnare* challenge: simultaneously challenged with *F. columnare* and *Nanophyetus salmincola* (Fc+cercariae), *F. columnare* (Fc) only, exposed to *N. salmincola* cercariae (C+cercariae) only, and a control. The difference in cumulative percent mortality between the Fc and Fc+cercariae groups on day 10 were not significant.

APPENDIX B: AVIRULENT *FLAVOBACTERIUM COLUMNARE* ISOLATE
PROTECTS AGAINST VIRULENT ISOLATES

Introduction

In December 2013, prior to the *F. columnare* challenge described in Chapter 3, a coinfection challenge of the same design was performed but with a different *F. columnare* isolate. However, no mortality was observed. Therefore, a subsequent challenge was performed 16 weeks later, to test if these fish would have protection against another *F. columnare* isolate.

Methods

Fish

Spring Chinook salmon (~1.5g) were obtained from Marion Forks fish hatchery (Idanha, OR, USA) in June 2013. These were from the same stock of fish as described in Chapter 3, they were maintained and treated as described above.

Snails

Freshwater *Juga* spp. snails were from the same stock as used in Chapter 3; these were maintained in 100 L flow-through tanks.

Bacteria and culture conditions

The *F. columnare*, designated CHSDX13, originally used for the first exposure was isolated in 2013 from the kidney of a juvenile Chinook salmon used as a sentinel fish placed downstream from the Dexter Ponds facility. The *F. columnare* isolate (DD3) used for the subsequent challenge was described above in Chapter 3. Aliquots of both isolates were mixed with 20% glycerol and maintained at -80˚C until used for bacteria challenges. All culture conditions and media for *F. columnare* were the same as described previously in Chapter 3.

The LD_{50} for the CHSDX13 isolate was determined by challenging duplicate groups of 20 fish in 25 L tanks with dilutions of a *F. columnare* culture at 0.1 and 0.5 OD_{540} using a 1 h static immersion challenge at 18°C. The LD_{50} for this *F. columnare* isolate corresponded to the 0.50 OD_{525} and was achieved by diluting 60 mL of 1.0 OD_{540} culture in 250 mL of tank water before adding directly to the tank at a 1:40 dilution.

Nanophyetus salmincola exposure

N. salmincola exposures were conducted as described in Chapter 3.

Initial F. columnare challenge

After *N. salmincola* exposure, fish were randomly assigned to 25 L tanks ($n = 30$ fish/tank) with a 2 X 2 factorial design, in which *N. salmincola*-parasitized or control fish were challenged with either *F. columnare* or sterile TYI broth. Treatments were replicated three times for a total of 12 tanks. Treatments were designated as control (C), parasitized by *N. salmincola* (N), challenged with *F. columnare* (Fc), and parasitized by *N. salmincola* and challenged with *F. columnare* (N+Fc; coinfection). After transfer, the water temperature was gradually increased from 13°C to 18°C over a few hours. Fish were acclimated to this temperature for 1 week prior to *F. columnare* challenge. Fish in the Fc and N+Fc treatments were challenged with *F. columnare* with interrupted water flow for 60 min by adding a 1:40 dilution (v/v) of a 0.5 OD_{540} culture to each tank. Fish in the C and N treatments were challenged in the same manner with diluted, sterile TYI broth.

Second challenge

After 3 months, the same challenge procedure as in the first challenge was repeated using the DD3 isolate of *F. columnare*.

Third challenge

Two weeks after the second *F. columnare* challenge, the fish were randomly reassigned to new 25 L tanks (*n* = 25 fish/tank) keeping fish nested within the original treatment group, and were designated "veterans" as they had previously been challenged with *F. columnare*. A second set of naïve *N. salmincola*-parasitized and control fish were added as a separate treatment set that had not had been challenged with *F. columnare*. Treatments were replicated three times for a total of 18 tanks. Treatments were designated as control (C), parasitized by *N. salmincola* (N), veteran and challenged with *F. columnare* (Fc), and veteran, parasitized by *N. salmincola,* and challenged with *F. columnare* (N+Fc), naive and challenged with *F. columnare* (naïveFc), and naive, parasitized by *N. salmincola,* and challenged with *F. columnare* (naïveN+Fc).

Fish monitoring

Fish monitoring, necropsy and tissue sampling, bacteria isolation, and *N. salmincola* metacercariae quantification was performed as described in Chapter 3.

Statistical analysis

Logistic regression models with a random intercept for tank were used to fit the cumulative percent mortality data from each challenge. A likelihood ratio test was used to evaluate if naïve group mortality differed from the veteran group mortality. Mean values were considered significantly different at $P < 0.05$.

Results

No mortality was observed in the first *F. columnare* challenge and the second challenge resulted in mortality in 12 of 360 fish. This was spread evenly across the Fc and N+Fc groups.

For the third challenge, cumulative percent mortality for both the naïve Fc groups (naïveN+Fc, naïveFc) was higher (73.3% ± 5.3, 64.0% ± 10.1) compared to the veteran Fc groups (45.6% ± 11.9, 41.6% ± 8.0). Most of the *F. columnare*-induced mortality occurred with 48 hours post challenge (Fig. B.1). No disease-related mortality was observed in C or N treatment groups (Fig. B.1).

Discussion

The lower mortality in the veteran group suggests that previous exposure to an avirulent *F. columnare* isolate may cause some protection, but this is not complete as mortality was still relatively high. There was a trend suggesting encysted *N. salmincola* affects *F. columnare* susceptibility in juvenile Chinook salmon, however, this was not conclusive. Any coinfection effect on mortality was seemingly negligible in the veteran groups, but more pronounced between the naïveN+Fc and naïveFc, suggesting there may be some parasite interaction. Due to the inadvertent design of these challenges, it is difficult to be conclusive as repeatability of this experiment is challenging.

The results of this challenge provide insights to how wild fish may react to different bacterial strains in the environment. As *F. columnare* is suggested to be ubiquitous in the environment (Kunttu *et al.* 2012), it may be that wild salmonids are more resistant to the bacteria.

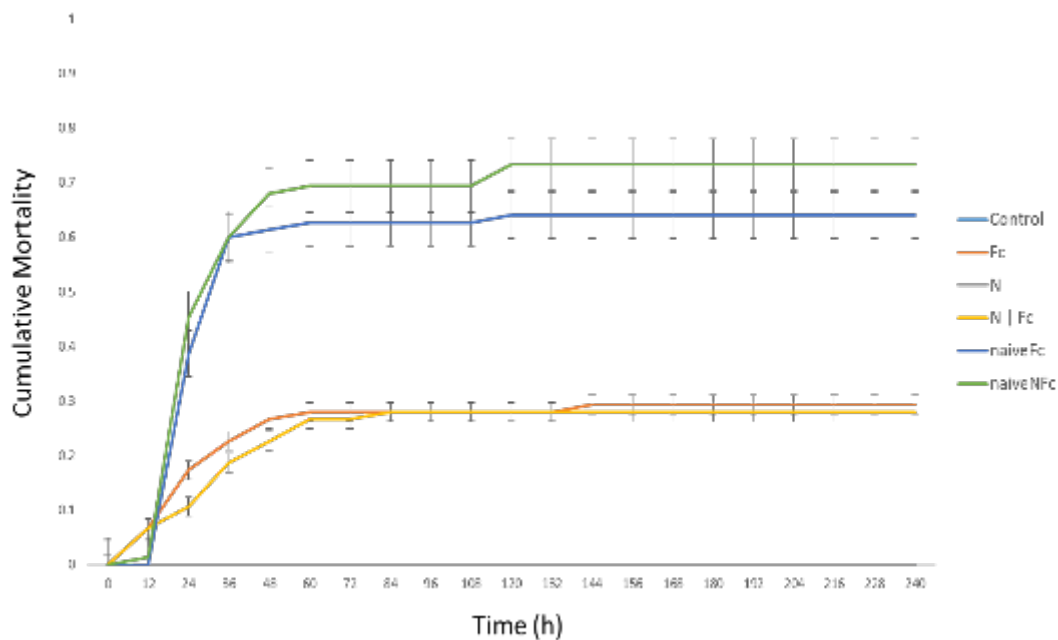Figure B.1. Cumulative percent mortality experienced by juvenile Chinook salmon in one of six treatments 240 h post *Flavobacterium columnare* challenge: naive challenged with *F. columnare* and *Nanophyetus salmincola* (naiveN+Fc), naïve *F. columnare* (naiveFc) only, veteran challenged with *F. columnare* and *Nanophyetus salmincola* (N+Fc), veteran *F. columnare* (Fc) only, parasitized with *N. salmincola* (N), and a control (C).

APPENDIX C: SUPPLEMENTARY MATERIAL FOR MACROPARASITE AND MICROPARASITE SURVEY OF RESIDENT FISHES IN UPPER WILLAMETTE TRIBUTARIES

Infectious agents may be maintained in an aquatic system through reservoir species. We surveyed the distribution of specific microparasites and macroparasites, including metazoan parasites, within wild resident fishes in three upper Willamette River tributaries. From 2011-2013, 8 different resident fish species were examined for one viral and four bacterial microparasites known to cause mortality in hatchery populations within the Willamette River to assess potential for non-salmonid pathogen reservoirs (Table C.1). The only detected microparasites were *Renibacterium salmoninarum* and *Flavobacterium columnare* which was detected at low prevalence (Table C.2). Low pathogen prevalence throughout the sample population suggests low prevalence within these populations. Metazoan parasite communities consisted of 23 identified species with no detection of highly pathogenic macroparasites. *Nanophyetus salmincola* was the most common macroparasite and was recorded in 5 of 8 fish species examined. Additional parasites commonly observed included: *Apophallus* sp., *Sanguinicola* sp., *Myxobolus insidiosus,* and *Salmincola californiensis*. Here, we report the survey results.

Table C.1. Number of fish collected at each site during each survey year

Species	MFW			Site Marion Forks			MCK			SS		
	2011	2012	2013	2011	2012	2013	2011	2012	2013	2011	2012	2013
Chinook Salmon (*Oncorhynchus tshawytscha*)		13	1		13†		48	74	108		35	
Steelhead/ Rainbow Trout (*O. mykiss*)		59	1		2		29	21	4		35	
Cutthroat Trout (*O. clarki*)		7			11	31	9	11	1		2	
Dace (*Rhinichthys* spp.)		6						2	1			
Northern Pikeminnow (*Ptychocheilus oregonensis*)		2						1				
Sculpin (*Cottus* spp.)		5	10		6	7		2	1		2	
Redside Shiner (*Richardsonius balteatus*)		16						1				
Threespine Stickleback (*Gasterosteus aculeatus*)								2				
Largescale Sucker (*Castostomus macrocheilus*)	6	1										
Mountain Whitefish (*Prosopium williamsoni*)		2			2			3			7	

Table C.2. Prevalence of select microparasites in 2012 and 2013. The values are the number of positive/the total number analyzed; positives are bold with percentages of infected individuals in parentheses. Sites are separated by season.

2012	Target Microparasites				
Season, subsite, and species of sample	*Flavobacterium columnare*	*Flavobacterium psychrophilum*	*Aeromonas salmonicida*	*Renibacterium salmoninarum*	IHNV
Summer (Jun – Aug)					
MFW					
Above Hatchery					
Chinook Salmon (n=2)	0	0	0	0	0
Rainbow Trout (n=7)[+]	0	0	0	**4/7 (57%)***	0
Below Hatchery					
Chinook Salmon (n=6)	0	0	0	**1/6 (17%)***	0
Rainbow Trout (n=32)	0	0	0	**1/32 (3%)***	0
Cutthroat Trout (n=3)	0	0	0	0	0
Dace (n=3)	0	0	0	0	0
Pikeminnow (n=2)	0	0	0	0	0
Sculpin (n=2)	0	0	0	0	0
Shiner (n=7)	0	0	0	0	0
Sucker (n=1)	0	0	0	0	0
Mountain Whitefish (n=2)	0	0	0	0	0

MCK

Above Hatchery

Chinook Salmon (n=10)	0	0	0	0	0
Cutthroat Trout (n=2)	0	0	0	0	0

Below Hatchery

Chinook Salmon (n=54)	0	0	0	0	0
Rainbow Trout (n=19)	0	0	0	**1/19 (5%)^**	0
Cutthroat Trout (n=9)	0	0	0	0	0
Dace (n=1)	0	0	0	0	0
Pikeminnow (n=1)	0	0	0	0	0
Sculpin (n=2)	0	0	0	0	0
Shiner (n=1)	0	0	0	0	0
Mountain Whitefish (n=3)	0	0	0	0	0

SS

Below Hatchery

Chinook Salmon (n=35)	0	0	0	0	0
Rainbow Trout (n=35)	0	0	0	**1/35 (3%)***	0
Cutthroat Trout (n=9)	0	0	0	0	0
Sculpin (n=2)	0	0	0	0	0
Mountain Whitefish (n=7)	0	0	0	**1/7 (14%)***	0

Marion Forks

Above Hatchery

Rainbow Trout (n=1)	0	0	0	0	0
Cutthroat Trout (n=11)	0	0	0	0	0
Sculpin (n=2)	0	0	0	0	0

Below Hatchery

Chinook Salmon (n=13)[†]	0	0	0	0	0
Rainbow Trout (n=1)	0	0	0	0	0
Sculpin (n=4)	0	0	0	0	0
Mountain Whitefish (n=2)	0	0	0	0	0

Autumn
(Sept – Nov)

MFW

Below Hatchery

Chinook Salmon (n=7)	0	0	0	0	0
Rainbow Trout (n=27)	0	0	0	0	0
Cutthroat Trout (n=4)	0	0	0	3/4 (75%)*	0
Dace (n=3)	0	0	0	0	0
Sculpin (n=3)	0	0	0	0	0
Shiner (n=9)	0	0	0	0	0

MCK

Above Hatchery

Chinook Salmon (n=10)	0	0	0	3/10 (30%)*	0
Rainbow Trout (n=2)	0	0	0	1/2 (50%)*	0
Dace (n=2)	0	0	0	0	0
Stickleback (n=2)	0	0	0	0	0

2013		Target Microparasites			
Season, subsite, and species of sample	*Flavobacterium columnare*	*Flavobacterium psychrophilum*	*Aeromonas salmonicida*	*Renibacterium salmoninarum*	IHNV
Autumn (Sept – Nov)					
MFW					
Above Hatchery					
Chinook Salmon (n=64) [+]	0	0	0	**61/64 (95%)***	0
Below Hatchery					
Chinook Salmon (n=1)	0	0	0	0	0
Rainbow Trout (n=1)	0	0	0	0	0
Sculpin (n=11)	**3/11 (27%)**	0	0	0	0

* Positive samples for *R. salmoninarum* were detected through ELISA, which was only performed on fish large enough to recover sufficient tissue volume.
^ Positive samples for *R. salmoninarum* were detected through DFAT, which was only performed on smaller kidney tissue volume.
† These fish were most likely unclipped, hatchery escapees.
[+]Adult salmonids received from a gillnet project above Dexter (Lookout Point Reservoir).