FACTORS AND PITFALLS INFLUENCING THE DETECTION OF BACTERIAL KIDNEY DISEASE

By

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A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

Fisheries and Wildlife – Doctor of Philosophy

2014

ABSTRACT

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Bacterial kidney disease (BKD) has been a significant problem for over 50 years in the Great Lakes basin (GLB) where it has been associated with large-scale fish mortalities and chronic infections in feral and propagated fish. The most commonly used and widely accepted method for detection of *R. salmoninarum* is a sandwich enzyme-linked immunosorbent assay (ELISA) on lethally collected kidney and spleen samples. While ELISA is relatively fast and more efficient than other serological assays, there are some concerns regarding what could be overlooked as a result of conducting a single assay on specific samples. Herein, I describe several factors that could affect the detection of BKD in feral and experimentally infected fish that are often overlooked upon assay or sample choices. Using data from the past decade, a decline in the overall presence of BKD in feral and hatchery-raised Chinook salmon (*Oncorhynchus tshawytscha*) (CHS), coho salmon (*O. kisutch*), and steelhead (*O. mykiss*) was observed. This also coincided with the implementation of enhanced biosecurity measures at state hatcheries and gamete-collection. Moreover, to potentially reduce the dependency on lethally collected samples for regular screening, the detection of *R. salmoninarum* in other sample types, such as mucus, blood, and a urine/feces mixture, was evaluated. All sample types were collected from experimentally infected CHS and compared for their efficiency to detect the presence of *R. salmoninarum* by standard bacterial culture techniques, nested polymerase chain reaction (nPCR), and sandwich ELISA. It was found that the urine/feces mixture was the preferred non-

lethal sample, and that a combination of assays and samples greatly increased the likelihood of detecting *R. salmoninarum*. Not only does collecting several samples enhance the detection of *R. salmoninarum*, but the presence of *R. salmoninarum* in the urine/feces mixture suggests that the bacterium may utilize the anal opening as a portal of entry. To determine if anti-*R. salmoninarum* antibodies are elicited during an infection, and to establish a protocol for their detection, a single-dilution indirect-ELISA was modified and assessed for its usefulness in detecting and semi-quantifying levels of elicited antibodies. The ability of the indirect-ELISA to detect antibodies was evaluated in several groups of experimentally infected rainbow trout and feral *Oncorhynchus* spp. The antibody response observed in the experimentally infected fish suggested their response was relatively short-lived. On the contrary, data from the spawning adult fish provided evidence of elevated antibodies, implying that they have been exposed multiple times to *R. salmoninarum* while existing in the GLB. A thorough analysis of *R. salmoninarum* infections was also conducted on over 600 CHS returning to spawn at several GLB gamete-collecting weirs to assess infection levels, shedding, and potential disease progression. Results suggested that female fish could be more susceptible to *R. salmoninarum* infection, but males may still play a role in shedding of the bacterium. Low levels of circulating anti-*R. salmoninarum* antibodies observed in the late spawning CHS, along with high intensities of infection, are evidence that fish returning to spawn later in the season are more heavily infected with *R. salmoninarum* than those early in the season. To this end, the detection of *R. salmoninarum* appears to depend upon several factors (i.e., type of sample and assay, time of collection, and fish age) that should be taken into consideration before deciding how to diagnose BKD in a particular fish population.

This dissertation is dedicated to my two loves, Jay and Cohen.

ACKNOWLEDGEMENTS

I would first like to give my sincere thanks to my major advisor, Dr. Mohamed Faisal, for his patience, first and foremost, as well as the guidance, wisdom, and life lessons that he has shared with me these past 10 years. I will never forget our first meeting at Lake St. Clair, and I owe my desire to educate myself as far as I have to him and his unwavering encouragement, for he saw something in me that I never did. Without his careful direction during my graduate career, I would not have been able to be as successful as I was. I thank him for his confidence in me, his support, and his direction, and I will always consider him my mentor and my friend.

In addition, I would like to genuinely thank my committee members: Dr. Travis Brenden, Dr. Kim Scribner, and Dr. Amber Peters. I am extremely grateful for each members' unique contributions and assistance they provided to my dissertation, as well as their advice and support throughout this process.

Many thanks are due to past and current members of the Michigan State University - Aquatic Animal Health Laboratory for their invaluable assistance throughout my research, as well as their everlasting friendship. Specifically, I would like to thank Michelle Gunn, Dan Bjorkland, Dr. Thomas Loch, Dr. Andrew Winters, Dr. Robert Kim, Dr. Elena Millard, Danielle Van Vliet, Elizabeth Throckmorton, Isaac Standish, and so many more!

My research efforts would not have been possible without the funding and support of the United States Fish and Wildlife Service (Grant #USFWS 30181AG013) and the Michigan Department of Natural Resources for providing fish used in the experimental challenges in this study.

Lastly, with my deepest gratitude, I would like to thank my family. To my parents, Paul and Robin, and my siblings, Jennifer, Juan, Emma, and Jack, thank you for being so supportive, even when it meant not seeing me (or hearing from me) for long periods of time. You are the best family I could ask for and I look forward to spending more time with you all. And most of all, thanks to the love of my life, my partner in adventure, my fiancé Jay, who I have needed by my side more than anyone throughout this process. Words cannot describe how excited I am to start my life with you.

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Introduction

Bacterial kidney disease (BKD), caused by the Gram-positive bacterium *Renibacterium salmoninarum*, is a systemic disease that causes chronic infections and mortalities in wild and aqua-cultured salmonid fish populations worldwide (Paterson et al., 1979; Evelyn et al., 1981; Holey et al., 1998). *Renibacterium salmoninarum* is an obligate intracellular pathogen that invades phagocytic cells (e.g., macrophages), where its presence is concealed from the fish's immune system, allowing it to proliferate (Gutenberger et al., 1997). Most notably, *R. salmoninarum* secretes excessive amounts of a water-soluble cell surface 57 kilodalton heavily glycosylated protein (p57) that enables the bacterium to spread easily throughout the fish host. These unique characteristics of *R. salmoninarum* permit the bacterium to survive within the fish host relatively undetected for long periods of time, resulting in chronic infections.

While *R. salmoninarum* has been detected in non-salmonids, it has a predilection for the kidneys of many salmonid species (MacLean and Yoder, 1970; Mitchum et al., 1979; Traxler and Bell, 1988). In the Great Lakes basin (GLB), salmonids play a critical role in the ecosystem, as they are one of the top predators and are a large part of a $7 billion per year fishing industry (Tsehaye et al., 2014). Since the 1970s, the Michigan Department of Natural Resources (MDNR) has been aiming to reduce the presence and effects of BKD in the Great Lakes salmon populations through regular health inspections and multiple prevention and control measures. Routine disease screening is an effective way to detect *R. salmoninarum* in a population, yet the stage of infection a fish is experiencing and the subsequent positive or negative result depends on the detection method that is used. For example, a sandwich enzyme-linked immunosorbent

assay (ELISA) is a common method used for detection of *R. salmoninarum* in a kidney and spleen sample. ELISAs are preferred by many professionals because they can test many samples at once in a relatively short period of time. However, as most BKD ELISAs test for the presence of the p57 antigen, a positive result usually indicates an active or a recent infection, but does not elaborate on the status of infection. Using multiple diagnostic assays that inform on other characteristics of the bacterium (i.e., detection of nucleic acids, viable bacteria, or specific antibodies) and the resultant stage of infection could influence the management and treatment decisions made by fishery and hatchery biologists.

Furthermore, as the kidneys and spleen of fish remove circulating bacterial antigens from the host, they are commonly used to provide indication of an infection (Bruno and Poppe, 1996). However, little is known regarding the use of other tissues (e.g., mucus, blood, feces) for detection of the pathogen. However, fish must be euthanized in order to collect the necessary kidney and spleen samples to detect *R. salmoninarum*. Not only could tissues such as mucus, blood, or feces reduce the need to sacrifice fish, but detection of *R. salmoninarum* in these tissues could also contribute to a better understanding of how the bacterium spreads in a fish host after infection.

By using a single diagnostic assay (e.g., ELISA, PCR, or bacterial culture), it is likely that the full range of infections (onset, established, active, recovering, and remission) will not be detected, thus potentially resulting in an underestimated prevalence of disease, which can impact treatment decisions for the fish population. Furthermore, as the sandwich ELISA detects the presence of the p57 antigen found on the outer surface of *R. salmoninarum*, it reveals whether a fish has been exposed to the bacterium, but not if the fish is producing antibodies. It

would be highly beneficial to know if the fish was mounting an immune response, and therefore would not require treatment with antibiotics. Lastly, sacrificing fish to collect a kidney and spleen sample for BKD testing can be extremely difficult for valuable broodstocks and endangered species. To this end, the overall objective of this study was to evaluate the various shortcomings and assets of currently used and new diagnostic assays and sample types for the detection of *R. salmoninarum*.

Firstly, a thorough review of literature regarding the history, characteristics, geographical and host distribution, diagnostic methods for detection, and prevention and control measures for BKD is given in Chapter 1.

Chapter 2 of this study evaluated the implementation of several enhanced biosecurity measures conducted by the state of Michigan at their hatcheries and gamete-collection weirs. Substantial declines in the overall prevalence of BKD were detected in each of the examined fish stocks, with differences in the prevalence of BKD observed in the different species and strains.

In Chapter 3, in addition to kidney and spleen samples, other samples types (mucus, blood, and a urine/feces mixture) were also tested by molecular, serological, and standard bacterial techniques to assess their ability to detect BKD, taking the effects of exposure route and the resultant disease course into consideration. Additionally, the number of fish that would be required to sample non-lethally was investigated. Analyses suggested that the urine/feces mixture had the best potential to detect *R. salmoninarum* compared to the mucus or blood. Detection was further enhanced when the urine/feces mixture and the kidney and spleen sample were tested in conjunction.

In Chapter 4, a single-dilution indirect ELISA was evaluated with the aim of detecting anti-*R. salmoninarum* antibodies in experimentally infected and feral *Oncorhynchus* spp. All groups of experimentally infected and feral fish were found to produce detectable levels of antibodies, although there were minimal differences among them. While the feral fish produced elevated levels of antibodies, the observed antibody response in experimentally infected fish was short-lived and long-term elevated levels of antibody production were not observed.

Chapter 5 investigated the prevalence and intensity of *R. salmoninarum* infections in male and female Chinook salmon returning to spawn at multiple gamete-collecting weirs in Michigan using several detection methods. Also, potential diagnostic patterns indicative of a progressive *R. salmoninarum* were considered. A greater prevalence of BKD infection was observed at the Swan River Weir, with female Chinook salmon appearing to be more susceptible to *R. salmoninarum* than males. Lastly, evidence of disease progression was observed, with earlier stages and more intense infections occurring later in the spawning run.

Lastly, the overall conclusions and recommendations for future research are presented in Chapter 6. This research highlights that despite the encouraging decline of BKD in the GLB over the past decade, there is still much we need to learn to maximize what is achieved with routine health inspections. Optimal opportunities to improve management and treatment decisions are being overlooked and neglected. More consideration needs to be taken with regards to how the chosen assay and sample type can affect the diagnosis of disease.

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Chapter 1

Literature Review

1. Historical background

Renibacterium salmoninarum is the causative agent of Bacterial Kidney Disease (BKD), a serious bacterial disease of salmonid fish species worldwide. Bacterial Kidney Disease was first described in 1930 in Atlantic salmon (*Salmo salar*) from the rivers Dee and Spey in Aberdeenshire, Scotland (Mackie et al., 1933; Smith, 1964). The disease was initially referred to as Dee Disease, and has also been known as Kidney Disease, Salmonid Kidney Disease, and Corynebacterial Kidney Disease (Fryer and Sanders, 1981). Granulomatous lesions, which are the characteristic clinical sign of BKD, were observed in the spleen and other internal organs of Atlantic salmon, along with a small gram-positive diplobacillus (Mackie et al., 1933). Shortly after the initial discovery of BKD in Scotland, a similar bacterial disease was described in brook trout (*Salvelinus fontinalis*), brown trout (*Salmo trutta*), and rainbow trout (*Oncorhynchus mykiss*) at a Massachusetts state fish hatchery (Belding and Merrill, 1935). Several external symptoms were observed, including exophthalmia, raised, fluid-filled blebs, abscesses, and reddening at the base of pectoral fins (Belding and Merrill, 1935). Granulomatous lesions similar to those described by Mackie et al. (1933) were noted in the kidneys, as well as in the livers and, to a lesser degree, in the spleens, along with excess fluid in the peritoneal and pericardial cavities, and hemorrhagic ovaries and testes (Belding and Merrill, 1935). Again, small, short, gram-positive, diplobacilli bacteria were recovered from the lesions associated with diseased fish (Belding and Merrill, 1935).

Subsequently, BKD was discovered in British Columbia, Canada in 1937 (Evelyn, 1986), followed by detections in the Pacific Northwest in the late 1940s, where an unknown kidney

disease infecting sockeye salmon (*O. nerka*), Chinook salmon (*O. tshawytscha*), and coho salmon (*O. kisutch*) from state fish hatcheries in California, Oregon, and Washington was described, which was later confirmed to be BKD (Rucker et al., 1951; Earp et al., 1953). Bacterial kidney disease then spread to the Laurentian Great Lakes Basin (LGLB) in the 1950s with the introduction of salmonids and their eggs from the Pacific Northwest (Allison, 1958), and has since been detected in several wild and aqua-cultured salmonid and coregonid species throughout the LGLB (MacLean and Yoder, 1970; Holey et al., 1998; Beyerle and Hnath, 2002; Jonas et al., 2002; Faisal et al., 2012). It was first found in Michigan in 1955 in brook trout from two state fish hatcheries (Allison, 1958). Furthermore, reports have linked the disease to mortalities in wild salmonids from Canada (Paterson et al., 1979; Evelyn et al., 1981), and mass fish kills in the LGLB (Holey et al., 1998).

In 1968, BKD was detected in farmed Atlantic salmon from Iceland (Helgason, 1985), and then became widespread in several salmonid species in Europe, including Denmark (Lorenzen et al., 1997), Germany (Hoffman et al., 1984), Finland (Rimaila-Pärnänen, 2002), France (DeKinkelin, 1974), Italy (Ghittino et al., 1977), Norway (Jansson, 2002), Poland (Kozin´ska et al., 2001), Spain (Martínez-Millán, 1977), Sweden (Jansson et al., 1996), former Yugoslavia (Fijan, 1977), North America (United States of America and Canada; Souter et al., 1987), and Japan (Kimura and Awakura, 1977). The disease has also spread to Chile (Sanders and Barros, 1986) and it is widely accepted that BKD is prevalent in all parts of the world where wild or cultured salmonids exist.

2. The pathogen

2.1. Nomenclature and current classification

Initially, the causative bacterium of BKD was suggested to be a member of the genus *Corynebacterium*, based on the Gram stain properties and morphology (Ordal and Earp, 1956; Smith, 1964). However, further taxonomic analyses revealed several deviations from typical *Corynebacterium* characteristics, including the absence of mycolic acid, differences in the sugar and amino acid compositions of the peptidoglycan cell wall layer, as well as a different guanine plus cytosine (G + C) content of DNA (Sanders and Fryer, 1980). As such, the authors proposed that this bacterium forms a single species in the new genus *Renibacterium* and they identified the bacterium as *Renibacterium salmoninarum* (Sanders and Fryer, 1980). Furthermore, sequencing of the 16S ribosomal ribonucleic acid (rRNA) from *R. salmoninarum* and additional evaluation of the G + C content has placed the organism in the order Actinomycetales and family Micrococcaceae (Banner et al., 1991; Gutenberger et al., 1991). *Arthrobacter* and *Micrococcus* spp. are the closest relatives to *R. salmoninarum* (Holt et al., 2001).

2.2. Cell morphology

Renibacterium salmoninarum is a short coccobacilli (0.3-1.5 by 1.0-1.5 µm), Gram-positive, non-sporing, non-capsulated, non-motile, and non-acid fast bacterium that is arranged singly, in pairs (diplo) and rarely as short chains (Sanders and Fryer, 1980; Fryer and Lannan,

1993). *Renibacterium salmoninarum* consists of two regions: a central region with lightly stained filaments (generally regarded to represent DNA) and a peripheral region filled with small, electron dense ribosomes (Young and Chapman, 1978).

2.3. Isolation, culture, and cultural characteristics

Renibacterium salmoninarum is a slow-growing, fastidious organism which has been difficult to isolate on ordinary bacterial media (Sanders and Fryer, 1980). Earp et al. (1953) initially cultured the bacterium from infected kidney tissues on an artificial medium that consisted of fish extract, glucose, yeast extract, and meat infusion in agar. The authors achieved limited growth with the first appearance of colonies after more than two weeks of incubation (Earp et al., 1953). Also, when the same authors used minced chick embryo tissues embedded in 1% agar or Dorset's Egg medium, they achieved better growth. Addition of 0.05 to 0.1% L-cysteine hydrochloride (HCl) to the Dorset's Egg medium has further enhanced the growth of *R. salmoninarum* upon primary isolation (Ordal and Earp, 1956). Moreover, Ordal and Earp (1956) developed the Kidney Disease Medium 1 (KDM1) which consisted of: tryptose 1.0%, beef extract 0.3%, NaCl 0.5%, yeast extract 0.05%, L-cysteine-hydrochloride 0.1%, human blood 20%, and agar 1.5%. This medium is also referred to as Cysteine Blood Agar Medium (Ordal and Earp, 1956). Additionally, Wolf and Dunbar (1959) achieved fair growth on Mueller-Hinton medium, also supplemented with cysteine.

Kidney Disease Medium 1 was later modified by Evelyn (1977) by replacing the human blood, tryptose, and beef extract with 20% fetal bovine serum and peptone, which was then

designated as KDM2. Furthermore, to reduce the time needed for primary isolation, which can be up to 6 weeks, Evelyn et al. (1989) added 25 µl of heavy inoculum of *R. salmoninarum* culture (commonly referred to as "nurse culture") to the center of the KDM2 plates, which can accelerate the growth in primary cultures. Evelyn et al. (1990) were able to again improve upon this method and achieve more consistent growth of the primary culture by replacing the nurse culture with 25 µl of filter-sterilized *R. salmoninarum* spent medium. The major drawbacks of KDM2 medium, however, are the high cost and presence of serum proteins, which hinder the identification of proteins of bacterial origin.

There are a number of serum-free media for *R. salmoninarum* growth that have been developed. Embley et al. (1982) developed a serum-free, semi-defined growth medium that supported secondary, but not primary, growth of *R. salmoninarum*. Also, Daly and Stevenson (1985) formulated the Charcoal Agar Medium in which they substituted activated charcoal for serum. Starliper et al. (1998) compared the performance of 13 serum-free media and 1 serum-supplemented media for the growth of *R. salmoninarum* isolates and found that there were no significant differences among the 14 medium formations when mean cell counts were compared after 10, 20, and 30 days of incubation. Additionally, Austin et al. (1983) incorporated four antibiotics (cycloheximide, D-cycloserine, oxolonic acid, and polymyxin B) into the KDM2 medium to control growth of other bacterial species. Austin et al. (1983) also reduced the volume of fetal bovine serum from 20% to 10% and designated the medium Selective KDM (SKDM; Austin et al., 1983). These modifications significantly reduced bacterial contaminants, facilitating the growth of *R. salmoninarum* from clinical and environmental samples (Austin et al., 1983). Lastly, Faisal et al. (2010) proposed modifying the SKDM to

incorporate 1% spent medium into the agar to enhance the growth of *R. salmoninarum* colonies, shorten the period of incubation, and minimize the growth of contaminating bacteria. This growth medium is known as Modified Kidney Disease Medium (MKDM) and is the only one to incorporate all of the improvements of the other media, cysteine, spent nurse medium, and antibiotics, into one medium (Faisal et al., 2010).

Renibacterium salmoninarum does require cysteine as an ingredient in the growth medium and grows best at 15°C, slowly at 5 or 22°C, and does not grow at all above 37°C (Smith, 1964). While colonies can develop as early as 14 days post-inoculation, up to 19 weeks may be required for isolation of very low numbers of bacteria (Benediktsdóttir et al., 1991). *Renibacterium salmoninarum* colonies are creamy (non-pigmented), shiny, smooth, round, raised, entire, and 1-2 mm in diameter (Austin and Austin, 2007). Additionally, on cysteine supplemented solid media, old colonies (i.e., >12 weeks) can appear extremely granular due to crystallization of cysteine (Austin and Austin, 2007).

2.4. Preservation of cultures

Several methods have been used to preserve different species of the order Actinomycetales, including *Streptomyces*, *Actinomyces*, and *Renibacterium* species. For long term preservation, methods such as lyophilization (Hopwood and Ferguson, 1969) and storage under liquid nitrogen (Pridham and Hesseltine, 1975) have been successfully used. Bacterial cells can also be preserved in diluted glycerol (10-20% v/v) and frozen at -20°C; however, repeated thawing and freezing cycles can affect the stability and viability of the cell (Wellington

and Williams, 1979). To overcome this drawback, Feltham et al. (1978) stored bacteria on glass beads in 10% (v/v) glycerol at -76°C. The glass beads allowed removal of small samples without thawing the entire culture, which was advantageous for long-term preservation (Wellington and Williams, 1979). Also, smaller aliquots can be utilized to prevent repeated thawing and freezing of larger samples. Preservation of small inocula of *R. salmoninarum* in KDM2 (Evelyn, 1977) or peptone saline (Starliper et al., 1997) and storage at -80°C were also successfully used.

2.5. Biochemical characteristics

Renibacterium salmoninarum is cytochrome oxidase negative, catalase positive, proteolytic, and dependent upon the presence of L-cysteine-HCl for growth in artificial media (Sanders and Fryer, 1980; Austin and Austin, 2007). Interestingly, *R. salmoninarum* isolates from different sources are homogeneous in their biochemical characteristics (Austin et al., 1983; Goodfellow et al., 1985; Bruno and Munro, 1986a), but the results for a given test can vary depending upon the testing system used. Thus, *R. salmoninarum* is negative for the gelatinase and DNase reactions by the API-ZYM system (Goodfellow et al., 1985), but was positive by standard methods (Bruno and Munro, 1986a). The organism is also known to be β-hemolytic on media supplemented with blood (Bruno and Munro, 1986a). Additionally, *R. salmoninarum* can liquefy gelatin, degrade Tween (20-60), hydrolyze casein, and is negative for esculin hydrolysis, DNase, urease, nitrate reduction, phosphatase, methyl red, indole test, and the carbohydrate utilization test (Table 1.1).

2.6. Antibiotic susceptibility

Renibacterium salmoninarum isolates are sensitive to carbenicillin, cephaloridine, chloramphenicol, erythromycin, novobiocin, rifampicin, streptomycin, sulfamerazine, and tetracycline (Wolf and Dunbar, 1959; Austin and Rodgers, 1980; Austin, 1985; Goodfellow et al., 1985). *Renibacterium salmoninarum* is also sensitive to enrofloxacin (Hsu et al., 1994), tiamulin, cefazolin (Bandin et al., 1991), and azithromycin (Rathbone et al., 1999). Furthermore, the organism is resistant to D-cycloserine, oxolonic acid (4 µg/ml), polymyxin B, and cycloheximide (Wolf and Dunbar, 1959; Goodfellow et al., 1985). It should be noted that while some studies have reported sensitivity of *R. salmoninarum* to chloramphenicol, it has also been shown to be not as effective (Austin, 1985).

2.7. Antigenic characteristics and virulence factors

Renibacterium salmoninarum is an intracellular, obligate pathogen that is able to invade all types of fish cells, in particular, the phagocytic cells (i.e., macrophages; Gutenberger et al., 1997; Ellis, 1999). The capability of *R. salmoninarum* to invade phagocytes or other cells depends upon certain virulence factors (Gutenberger et al., 1997; Ellis, 1999; Piganelli et al., 1999). It has been demonstrated that *R. salmoninarum* secretes a number of extracellular products (ECP) that possess proteolytic, hemolytic, and DNA degradation activities *in vitro* (Austin and Rodgers, 1980; Bruno and Munro, 1986a). Furthermore, a 65 kiloDalton (kDa) *R. salmoninarum* zinc metalloprotease-like protein has been extracted from *R. salmoninarum* ECP

that possesses hemolytic activities against a number of fish and mammalian erythrocytes. The encoding gene of the *R. salmoninarum* ECP with hemolytic activity was designated as hly (Grayson et al., 1995). Most notably, *R. salmoninarum* secretes a water-soluble, heat-stable, hydrophobic cell surface 57 kDa protein (p57) that is believed to be the major virulence determinant of this bacterium (Getchell et al., 1985). *In vitro*, purified p57 exhibited both hemolytic (Daly and Stevenson, 1990) and leuco-agglutinating (Wiens and Kaattari, 1991) properties. Moreover, *R. salmoninarum* is able to secrete excessive amounts of the p57 protein, enabling the bacteria to easily spread throughout the host (Evenden et al., 1993). Also, Hamel (2001) reported that *R. salmoninarum* isolates differed in their pathogenicity to salmonids, a finding that correlated positively with the amount of surface-associated p57.

The agglutinating properties of *R. salmoninarum* also contribute to its pathogenicity. Challenge of susceptible fish with auto-agglutinating strains of *R. salmoninarum* caused significantly higher mortality than non-auto-agglutinating strains (Daly and Stevenson, 1990; O'Farrell et al., 2000). Additionally, soluble *R. salmoninarum* surface proteins possess immunosuppressive action against the salmonid specific antibody response (Turaga et al., 1987), which was attributed not only to the p57 protein, but also to a 22 kDa surface protein (Fredriksen et al., 1997). Starliper et al. (1997) compared a number of strains of *R. salmoninarum* isolated from Chinook and coho salmon from the Pacific Northwest and Great Lakes regions in North America for virulence. Those authors found that virulence differed significantly among the isolates and concluded that isolates retrieved from Lake Michigan weirs were the most virulent compared to the Pacific Northwest (Starliper et al., 1997). Interestingly, while strains of *R. salmoninarum* from Lake Michigan are considered to be more virulent,

Chinook salmon from Lake Michigan are more resistant to *R. salmoninarum* infections than Chinook salmon from Green River, Washington (Purcell et al., 2008).

2.8. Molecular and genetic diversity

Although the biochemical uniformity and phylogenetic homology of *R. salmoninarum* strains are fairly similar (Bruno and Munro, 1986a; Gutenberger et al., 1991), a minimal molecular diversity was detected among strains isolated from different parts of the world (Alexander et al., 2001). Using polymerase chain reaction (PCR) amplification of length polymorphisms in the tRNA intergenic spacer regions, Alexander et al. (2001) succeeded in differentiating among isolates of *R. salmoninarum* from several farms in the United Kingdom. Moreover, genetic diversity was also detected among 40 North American isolates using the multilocus enzyme electrophoresis assay (Starliper, 1996). The highest genetic diversity was detected in strains isolated from Chinook and coho salmon returning to spawn at the Little Manistee River weir in Michigan (Starliper, 1996).

3. The disease

3.1. Disease course

Even though BKD develops slowly in host organisms, there are several factors that can influence the progression of BKD, including water temperature (Sanders et al., 1978; Fryer and

Sanders, 1981; Bullock and Herman, 1988), host factors (Evenden et al., 1993), and *R. salmoninarum* strain virulence (Starliper et al., 1997), which will be discussed later. Moreover, various disease signs may be overt or more obscure in individuals, depending on the extent of the infection.

3.1.1. External signs

Affected fish can manifest behavioral changes, as well as a wide range of external lesions that might vary according to the age and species of the fish affected and the virulence of the *R. salmoninarum* strain (Fryer and Sanders, 1981; Bullock and Herman, 1988; Evenden et al., 1993). Superficial blebs (or blisters) of the skin, exophthalmia, erratic swimming behavior, deep abscesses all over the body surface, and cavitations in muscles have been reported in affected fish (Belding and Merrill, 1935; Smith, 1964; Fryer and Sanders, 1981; Bullock and Herman, 1988; Evenden et al, 1993). The blebs and cavitations may contain a white to yellowish or hemorrhagic fluid (Bullock and Herman, 1988). Petechial hemorrhages in muscles and fins and ascitic fluid in the peritoneal cavity have also been reported (Belding and Merrill, 1935; Earp et al., 1953; Evelyn, 1993). In very rare cases, the external signs of the disease in coho and Chinook salmon might only be manifested by exophthalmia, associated with the accumulation of infective fluid containing large amounts of the bacteria, pus, and necrotic tissue in the enlarged eyes (Bullock and Herman, 1988).

3.1.2. Internal lesions

The kidneys of affected fish usually exhibit white foci that contain leukocytes, bacteria, and host cell debris and can be swollen (Fryer and Sanders, 1981). In advanced cases, the spleen may increase in size, the liver can appear very pale in color, and the kidneys are mostly grayish in color (Wood and Yasutake, 1956; Fryer and Sanders, 1981; Evenden et al., 1993). The most characteristic clinical lesions associated with BKD are the presence of scattered nodules of various sizes over the surface of the kidneys, spleen, and liver (Belding and Merrill, 1935; Snieszko and Griffin, 1955; Klontz, 1983). In some instances, petechial hemorrhages were noticed in the muscles lining the peritoneum, often associated with the accumulation of ascitic fluid (Ferguson, 1989). An opaque pseudomembrane covering the internal organs has also been reported, especially in fish maintained at a temperature below 9°C (Snieszko and Griffin, 1955; Bell, 1961; Fryer and Sanders, 1981). The pseudomembrane consists of fibrin and leukocytes (Smith, 1964). Similar membranes occur in trout at higher temperatures (12-13°C; Bullock and Herman, 1988). Hemorrhages with a white or yellow viscous fluid in the hindgut and petechial hemorrhages were often found in the peritoneum of infected Atlantic salmon (Smith, 1964).

3.1.3. Histopathology

The initial histopathological description indicated that the kidneys were the major organ affected by *R. salmoninarum* infection (Belding and Merrill, 1935). Infected brook trout and

brown trout demonstrated microscopic lesions in the kidneys, and to a lesser extent, in the liver and spleen (Belding and Merrill, 1935). Lesions are typically chronic in nature, with multiple granulomas that resemble those observed in mammalian tuberculosis (Snieszko and Griffin, 1955; Wood and Yasutake, 1956). Fibrotic lesions have also been noticed in the kidneys, spleen, liver, and intestine of infected fish, with proliferating fibroblasts forming distinct nodules that then coalesce to form large masses of affected tissues (Wood and Yasutake, 1956).

It is believed that the granulomas are formed as a result of macrophage activation, followed by their adherence to each other, forming giant cells (Secombes, 1985). The giant cells and activated macrophages can release large amounts of lytic enzymes into the surrounding tissues, leading to necrosis at the central part of the granuloma (Bruno, 1986; Jansson, 2002). Interestingly, *R. salmoninarum* can occur extracellularly or intracellularly in the granulomas or necrotic foci (Bruno, 1986; Bullock and Herman, 1988). In the kidneys, the hematopoietic tissue of the anterior kidneys appears to be affected initially, followed by widespread damage to the excretory portion of the kidneys (Wood and Yasutake, 1956; Jansson, 2002). Kidney pathology can include hypercellularity of the glomeruli, occlusion of Bowman's space by filamentous or granular deposits, and presence of eosinophilic granules in proximal tubules (Young and Chapman, 1978; Sami et al., 1992). Massive myocarditis, meningitis, and encephalitis were also recorded in some salmonids (Wood and Yasutake, 1956; Speare, 1997). In the liver, histopathological changes take the form of granulomatous nodules in the connective tissue stroma between the cords of the hepatic cells (Wood and Yasutake, 1956).

3.2. Host susceptibility

There are a number of reports demonstrating that salmonid species can differ in their susceptibility to BKD. Generally, Chinook salmon, coho salmon, and domestic Atlantic salmon are considered to be the most susceptible to BKD and are more likely to experience mortality due to the disease. Additionally, lake trout (*Salvelinus namaycush*), rainbow trout [steelhead], brook trout, lake whitefish (*Coregonus clupeaformis*), and bloater (*C. hoyi*) are only somewhat susceptible to BKD and may not necessarily experience mortality upon becoming infected (Starliper et al., 1997; Jonas et al., 2002; Hay, 2003; Nuhfer et al., 2005).

In addition to differences between species, different strains of the same species have also been observed with differential susceptibility to BKD. For example, coho salmon of three different transferrin genotypes (AA, AC, and CC) differed in resistance to experimental infection with *R. salmoninarum* (Suzumoto et al., 1977). Furthermore, three populations of Chinook salmon from different rivers demonstrated various mortality rates when exposed to *R. salmoninarum* in an experimental infection (Beacham and Evelyn, 1992). Winter et al. (1980) reported similar results in coho salmon and steelhead. Further, Belding and Merrill (1935) reported that brook trout were more susceptible to *R. salmoninarum* infection than the rainbow trout when experimentally infected. In this context, Mitchum and Sherman (1981) also reported that brook trout were more susceptible to natural BKD infections than rainbow trout and brown trout.

3.3. Pathogenesis and immunity

3.3.1. Process of infection and pathogenesis

Renibacterium salmoninarum has the ability to induce uptake by non-phagocytic cells and can also survive ingestion, providing a possible means of entry into the host via the gastrointestinal tract (Balfry et al., 1996; Evelyn, 1996; Flaño et al., 1996). The gills are a less likely portal of entry for *R. salmoninarum*, as it has been demonstrated that *R. salmoninarum* is not internalized by healthy rainbow trout gills *in vitro* (McIntosh et al., 2000). Uptake of *R. salmoninarum* through the eggs and reproductive fluids from parents to offspring has been demonstrated in several vertical transmission studies (Evelyn et al., 1984; Evelyn et al., 1986a, b; Bruno and Munro, 1986b).

Renibacterium salmoninarum is considered to spread through blood and also through intracellular habitation and replication in macrophages (Gutenberger et al., 1997; Ellis, 1999). Even though *R. salmoninarum* is a slow-growing organism, once it is established in the circulatory system, it can reach levels of 10^9 cells/g in spleen and kidney tissues before initiation of fish mortality (Evelyn, 1996). With most pathogens, opsonization of the pathogen by an antibody and/or complement usually limits the ability of the pathogen to cause harm; however, with *R. salmoninarum*, opsonization actually increases its ability to survive and replicate within phagocytes (Bandin et al., 1995). To survive and replicate, *R. salmoninarum* needs to acquire nutrients from the host. In the absence of iron, *R. salmoninarum* may produce iron reductase, which makes bound iron more available for bacterial uptake (Grayson et al., 1995).

Renibacterium salmoninarum also produces large amounts of the p57 antigen (Wiens and Kaattari, 1989), both in serum and intracellularly. The quantity it produces can neutralize the vast majority of antibodies that may be evoked in response to infection; therefore, these antibody-p57 complexes may remain in tissue and contribute to tissue destructive hypersensitivity and result in granulomas (Bruno, 1986; Sami et al., 1992). Moreover, the p57 protein has immunosuppressive and tissue destructive properties. It can agglutinate salmon leukocytes and suppress antibody production against unrelated antigens *in vitro*, thereby further reducing the protective capabilities of the host (Turaga et al., 1987; Wiens and Kaattari, 1991). The p57 is a potent inhibitor of the phagocyte respiratory burse response, which typically plays a critical role in degrading bacteria (Campos-Perez et al., 1997). Furthermore, p57 could potentially decrease the bactericidal activity of juvenile Chinook salmon macrophages against another important fish pathogenic bacterium, *Aeromonas salmonicida* (Siegel and Congleton, 1997).

Lastly, Senson and Stevenson (1999) suggested that p57 and its breakdown products might form a protective layer around *R. salmoninarum* cells. Cell surface associated p57 and its breakdown products could effectively block highly immunogenic areas of the bacterial cell surface from detection by the host's immune system (Wiens and Kaattari, 1999). Indeed, bacterial cells stripped of p57 induced a stronger immune response than those not stripped of p57 in an experimental challenge (Wood and Kaattari, 1996).

3.3.2. Effect of BKD on host immune response

Grayson et al. (2002) studied the immunosuppressive effect of *R. salmoninarum in vivo* and *in vitro*. Within the *in vitro* assay, macrophages showed a rapid inflammatory response in which the expression of inducible cyclooxygenase, inducible nitric oxide synthase, interleukin-1β (IL-1β), and major histocompatibility complex class II (MHC II) were enhanced (Grayson et al., 2002). Additionally, at first tumor necrosis factor-α (TNF-α) expression was greatly reduced, but then later increased (Grayson et al., 2002). In the *in vivo* study, intraperitoneal (i.p.) injection of *R. salmoninarum* DNA vaccine constructs reduced the expression of IL-1β, Cox-2, and MHC II, but stimulated TNF-α (Grayson et al., 2002). The authors concluded that the p57 suppresses the host immune response and proposed that the chronic granulomatous reaction is due to the prolonged stimulation of TNF-α (Grayson et al., 2002). Furthermore, the p57 possesses immunosuppressive abilities against the salmonid-specific antibody response (Turaga et al., 1987), tissue destructive properties (Bruno, 1986), and is also capable of agglutinating salmon leukocytes (Wiens and Kaattari, 1999).

Aside from its opsonizing action, antibodies can interact directly with free p57, creating immune complexes that accumulate within the tissue and cause hypersensitivity reactions, which can result in granulomas and tissue damage (Bruno, 1986). Macrophage activating factor (MAF)-activated macrophages can effectively kill *R. salmoninarum* cells, and the proliferation and action of T-cells in activating macrophages may be the primary successful immune response against *R. salmoninarum* (Secombes, 1985; Hardie et al., 1996). However, low temperatures can frequently suppress the production of MAF in immature helper T-cells,

thereby reducing the potential immune response against *R. salmoninarum* (Siegel and Congleton, 1997).

3.3.3. Environmental factors

3.3.3.1. Effect of diet

Research has suggested that the prevalence and severity of BKD might be partially associated with particular environmental and dietary factors. Diets formulated of gluten as opposed to cottonseed meal have resulted in higher BKD prevalence in several hatcheries in the state of Washington (Wood, 1974). Additionally, Wedemeyer and Ross (1973) demonstrated that while the incidence of a BKD infection was similar in fish fed gluten and cottonseed diets, the non-specific stress of the infection was more severe in the gluten group, as reflected by the increased ascorbate depletion, and could result in elevated mortalities. While Sakai et al. (1986) concluded that vitamins had no effect on BKD prevalence, Paterson et al. (1981) indicated that Vitamin A, zinc, and iron levels can be significantly reduced in BKD-infected fish. Furthermore, subsequent feeding trials demonstrated a lower incidence of BKD in fish fed diets high in trace elements (iron, copper, magnesium, cobalt, iodine, and fluorine) or low in calcium (Paterson et al., 1981). Lastly, Woodall and LaRoche (1964) suggested that iodine insufficiency could also be responsible for increased BKD incidence in juvenile Chinook salmon.

Limited food availability, in addition to other factors, may also be related to increased BKD infections (Holey et al., 1998). The Chinook salmon epizootics that occurred in Lake

Michigan in the late 1980s were attributed to a lack of a food source (i.e., alewives) and a co-infection of *R. salmoninarum* and the acanthocephalan *Echinorhynchus salmonis* (Holey et al., 1998). The lack of an abundant food source (i.e., alewives), which could not satisfy the energy demands of Chinook salmon, coupled with the presence of *E. salmonis* in the intestinal tract, likely resulted in nutritional stress that made the fish vulnerable to BKD (Holey et al., 1998).

3.3.3.2. Effect of temperature

BKD has been reported to occur over a wide range of water temperatures (Belding and Merrill, 1935; Earp et al., 1953; Fryer and Sanders, 1981; Bullock and Herman, 1988). Sanders et al. (1978) reported the occurrence of an earlier time-to-death (21-34 days versus 61-70 days) for experimentally infected juvenile coho salmon, sockeye salmon, and steelhead that were held at 15-20°C compared to 6.7°C. Similarly, Wood (1974) reported that mortalities due to BKD in hatcheries occurred 30-35 days post exposure at temperatures above 11°C, but took 60-90 days at 7.2-10°C. Moreover, Earp et al. (1953) detected seasonal trends occurring with BKD epizootics in Washington state hatcheries. Most of the epizootics occurred during the autumn and winter, under conditions of declining water temperatures; however, the greatest mortalities were associated with periods of the highest water temperatures. Also, it was noted that during periods of low water temperatures, the disease produced a slow steady death rate (Earp et al., 1953).

3.3.3.3. Effect of estuarine and salt-water environments

Despite the fact that BKD occurs mainly in freshwater, significant infections also occur in saltwater (Banner et al., 1983). Reports have demonstrated that deaths continued in Chinook, coho, and pink salmon (*Oncorhynchus gorbuscha*) stocks after movement to saltwater-rearing ponds (Earp et al., 1953; Bell, 1961). Additionally, Frantsi et al. (1975) reported that *R. salmoninarum* impaired the ability of Atlantic salmon smolts to acclimate to saltwater and caused subsequent reduction in their ocean survival. *Renibacterium salmoninarum* can also persist in individuals after spending prolonged time periods in saltwater (Ellis et al., 1978). Furthermore, Fryer and Sanders (1981) indicated that BKD was thought to be the main cause of death among coho salmon smolts released from the Siletz Hatchery in Oregon. These authors reported that the majority of deaths occurred between two and four months after the fish entered saltwater. Additionally, after migration into saltwater, fish continued to die due to BKD at an accelerated rate (Fryer and Sanders, 1981). Also, BKD infections can affect the ability of fish to acclimate to seawater and can result in death (Mesa et al., 1999). Further, Price and Schreck (2003) experimentally assessed the effect of BKD on saltwater preference of juvenile spring Chinook salmon and concluded that there was a significant negative relationship between mean infection level and saltwater preference. Their results demonstrated that the higher the level of BKD-infection, the less likely Chinook salmon were to migrate into saltwater or they took a much longer time to migrate permanently into saltwater (Price and Schreck, 2003). This behavior may increase the risk of avian predation for Chinook salmon migrating out to the ocean (Price and Schreck, 2003).

3.4. Epizootiology

3.4.1. Geographical distribution

Bacterial kidney disease has been reported worldwide, nearly everywhere that susceptible salmonid populations are present (Fryer and Sanders, 1981; Klontz, 1983). The disease is commonly reported in cultured salmonid species from North America, Europe, Japan, and South America (Fryer and Sanders, 1981; Bullock and Herman, 1988). Bacterial kidney disease has also been observed in a wide range of wild (Pippy, 1969; Evelyn et al., 1973; Ellis et al., 1978; Paterson et al., 1979; Mitchum and Sherman, 1981) and feral salmonid populations from North America (Elliott and Pascho, 1991; Sanders et al., 1992; Holey et al., 1998; Jonas et al., 2002; Faisal et al., 2012). The geographic range of BKD includes Canada, Chile, Denmark, England, France, Finland, Germany, Iceland, Italy, Japan, Norway, Poland, Scotland, Spain, Sweden, Turkey, United States, former Yugoslavia (Bullock and Herman, 1988; Lorenzen et al., 1997; Jansson, 2002). BKD was presumptively diagnosed and reported in Australia in the early 1970s in farmed Chinook salmon; however, further work identified the syndrome to be nocardiosis (Humphrey et al., 1987).

3.4.2. Host range

Renibacterium salmoninarum has been detected in Chinook salmon (Holey et al., 1998), coho salmon (MacLean and Yoder, 1970), brown trout, brook trout, rainbow trout (Belding and

Merrill, 1935; Mitchum et al., 1979), Pacific salmon, Atlantic salmon, lake trout (Awakura, 1978), grayling (*Thymallus thymallus*) (Kettler et al., 1986), lake whitefish and bloater (Jonas et al., 2002), and whitefish (*Clupeaformis lavretus*) in Finland (Rimaila-Pärnänen, 2002). The organism has also been detected in absence of disease in few non-salmonid species such as flathead (*Platycephalus indicus*), Pacific hake (*Merluccius productus*), and Pacific herring (*Glupea pallasi pallasi*) (Traxler and Bell, 1988; Kent et al., 1998). *Renibacterium salmoninarum* antigen has also been detected in Japanese sculpin (*Cottus japonicas*) and Japanese scallops (*Patinopecten yessoensis*) (Sakai and Kobayashi, 1992). Recently, the organism has been isolated for the first time from the adult parasitic stage of Lake Ontario sea lamprey (*Petromyzon marinus*) (Eissa et al., 2006). Experimental infections have shown that sablefish (*Anoplopoma fimbria*) are susceptible to *R. salmoninarum*, resulting in death in some instances, and can harbor the pathogen for up to 165 days post-infection, demonstrating their potential to act as reservoir hosts (Bell et al., 1990). Also, a natural outbreak of BKD in ayu (*Plecoglossus altivelis*) was documented in Japan, where they were cultured closely with masu salmon (*Oncorhynchus masou*) and were found to be more susceptible (Nagai and Iida, 2002).

3.4.3. Disease transmission

3.4.3.1. Source of infection

Renibacterium salmoninarum can survive in feces, saltwater, freshwater, and pond sediments for different durations of time. *Renibacterium salmoninarum* excreted in the feces

of clinically diseased trout can survive for up to one week in feces and two weeks in sterile seawater (Balfry et al., 1996). The organism can also survive in non-sterile freshwater and pond sediments for up to 21 days (Austin and Rayment, 1985). Thus, the oral-fecal route of horizontal transmission may contribute significantly to the increasing prevalence of BKD in salmonids.

3.4.3.2. Horizontal transmission

Several studies have demonstrated the ability of *R. salmoninarum* to be horizontally transmitted among fish. *Renibacterium salmoninarum* possesses a powerful capability of inducing uptake by tissue cells including the epithelial lining of the gastrointestinal tract (Bruno, 1986; Evelyn, 1996; Flaño et al., 1996). Infection is thereby likely to occur when sufficient numbers of bacteria are present within the immediate vicinity of an aquatic environment. The oral-fecal route of infection can also occur in net pens by ingestion of contaminated feces during feeding (Balfry et al., 1996). Waterborne infection has been shown to occur through the gills, eyes, lesions, and wounds (Evenden et al., 1993). *Renibacterium salmoninarum* was also transmitted by feeding fish infected or inefficiently pasteurized fish offal or fish flesh (Wood, 1974; Fryer and Sanders, 1981). Thus, uptake of *R. salmoninarum* through the intestinal wall is a likely pathway of infection (Jansson, 2002). Horizontal transmission can also occur between wild and stocked hatchery trout in natural systems (Mitchum and Sherman, 1981).

It has also been shown that coded wire tags, which are used extensively for identification and management of anadromous salmonid populations in the Pacific Northwest,

enhance the horizontal transmission of *R. salmoninarum* (Elliott and Pascho, 2001). Tagged fish demonstrated typical BKD lesions near the site of implantation. Furthermore, the authors suggested that the procedures might promote transmission of the pathogen among fish via contaminated tagging needles (Elliott et al., 2001).

3.4.3.3. Vertical transmission

Numerous studies have been conducted in the last several decades to study the role of vertical transmission of *R. salmoninarum* from mother to offspring via eggs. Allison (1958) was the first to report the development of BKD in offspring hatched at a facility where BKD had never been detected. Interestingly, the offspring were transferred as eggs from a hatchery where the disease had been endemic for many years (Allison, 1958). Bullock et al. (1978) demonstrated transmission of *R. salmoninarum* from parental broodstock to their progeny via the eggs. In some instances, the bacterium is believed to be located intra-ovum, within the perivitteline membrane of the egg, where it has been shown to be protected from surface disinfectants (Bruno and Munro, 1986b; Evelyn et al., 1986a, b; Evelyn, 1993). Coelomic fluid infected with high bacterial counts has also been shown to be an important source of infection for the egg (Evelyn, 1993). Moreover, intra-ovum infections can also occur prior to ovulation and directly from the ovarian tissue (Evelyn, 1993).

Contrary to the strong evidence for vertical transmission of BKD via female parental broodstock, there is little documentation in support of male contribution to vertical transmission. Evelyn et al. (1986b) reported that male coho salmon and steelhead did not play

a significant role in vertical transmission in experimental infections and Klontz (1983) submitted that the male does not contribute to vertical transmission either. However, Eissa et al. (2007) demonstrated similar levels of *R. salmoninarum* infection in milt and ovarian fluid from naturally infected brook trout and suggested that males do contribute to vertical transmission of BKD. Moreover, at certain gamete-collecting weirs in Michigan, Faisal et al. (2012) reported a higher incidence of *R. salmoninarum* infection in milt than ovarian fluid from naturally infected salmon, implying that males could be contributing to the vertical transmission of *R. salmoninarum*, although to what extent this is occurring is not known.

3.4.3.4. Fish as possible vectors and carriers

Although there is enough satisfactory data indicating that *R. salmoninarum* is an obligate intracellular pathogen of salmonid fishes, and the reservoir hosts and carriers of infection are other infected salmonids (Wood and Yasutake, 1956; Fryer and Sanders, 1981; Klontz, 1983; Bullock and Herman, 1988), there are still other indications regarding the possibility that non-salmonids can also act as a reservoirs or possible vectors for the bacterium. There are a few non-salmonid species that are able to contract the infection naturally or experimentally, and in turn, they have the potential to become carriers, playing an important role in transmission of the disease to salmonid species by cohabitation. For example, Pacific herring living in net pens with *R. salmoninarum*-infected coho salmon were also infected (Paclibare et al., 1988), as well as ayu living in close proximity to masu salmon in Japan (Nagai and Iida, 2002). Also, Pacific herring (Traxler and Bell, 1988), sablefish (Bell et al., 1990),

common shiner (*Notropis cornutus*) (Hicks et al., 1986), and fathead minnow (*Pimephales promelas*) (Hicks et al., 1986) were able to contract infection by i.p. injection of *R. salmoninarum*. The organism was also detected in moribund Pacific hakes (Kent et al., 1998). In addition, Japanese sculpin and flathead were also reported as possible vectors for the disease (Sakai and Kobayashi, 1992). More recently, the detection of *R. salmoninarum* from the adult parasitic stage of the sea lamprey suggests that it may also play a role in disease transmission (Eissa et al., 2006).

3.4.3.5. Possible vectors other than fish

A limited number of studies have been conducted investigating the assumption that animals other than fish can act as possible vectors for the transmission of *R. salmoninarum* to salmonid fish species. The Japanese scallop has been reported as a possible vector for *R. salmoninarum* transmission to coho salmon pen-raised in the neighboring seawater (Sakai and Kobayashi, 1992).

3.4.3.6. Reservoirs

While clinically infected, subclinically infected, or latent carrier salmonids are the main reservoirs of infection (Klontz, 1983; Richards et al., 1985; Bullock and Herman, 1988), bacterial laden-feces and *R. salmoninarum* rich pond sediment can also act as reservoirs of infection

(Austin and Rayment, 1985; Balfry et al., 1996). In addition, inefficiently pasteurized infected salmon viscera used as feed are a confirmed reservoir of infection (Wood, 1974).

4. Diagnosis of BKD

4.1. Type of sample used for pathogen detection

The currently accepted methods for detecting many bacterial fish pathogens, including *Renibacterium salmoninarum*, are outlined by the American Fisheries Society-Fish Health Section (AFS-FHS) Blue Book (2012) and the World Organisation for Animal Health (OIE) Manual of Diagnostic Tests for Aquatic Animals (2012). While these methods require the use of kidney and spleen samples, there are multiple tissues that can be used for *R. salmoninarum* detection.

4.1.1. Kidneys and spleen

The kidneys and spleen contain haematopoietic tissue and are major sites for blood filtration. Furthermore, they remove circulating bacterial antigens from the host and commonly provide indication of an infection (Bruno and Poppe, 1996). The kidneys and spleen are the recommended tissues used for detection of *R. salmoninarum* in salmonids by the AFS-FHS (2012) and the OIE (2012). Since the first detection of BKD, there have been numerous studies demonstrating the detection capability of the kidneys and spleen (Pippy, 1969; Bullock and Stuckey, 1975; Mitchum and Sherman, 1981; Pascho et al., 1991; Gudmundsdóttir et al.,

1993; Chase and Pascho, 1998; Faisal et al., 2012). While the kidneys and spleen are widely accepted as suitable sample types for BKD detection, they do require that the fish host be euthanized prior to collection.

4.1.2. Reproductive fluids

It is well established that *R. salmoninarum* is vertically transmitted intra-ovum and in ovarian fluid (Evelyn et al., 1986a). Due to this mode of transmission, *R. salmoninarum* is frequently detected in eggs and ovarian fluid from various salmonid species (Pascho et al., 1987; Pascho, et al., 1998; Rhodes et al., 1998; Eissa et al., 2007; Faisal et al, 2012). Additionally, while the extent to which they contribute to vertical transmission is unknown, *R. salmoninarum* has also been detected in milt from brook trout, Chinook salmon, coho salmon, and steelhead (Eissa et al., 2007; Faisal et al., 2012). Detection of *R. salmoninarum* in ovarian fluid has become an important management strategy to prevent vertical transmission (Pascho et al., 1991; Faisal et al., 2012), yet ovarian fluid may not always be readily available from each fish and can only be obtained from females.

4.1.3. Blood

Blood contains specific antibodies that have been produced to contest a particular pathogen (Alcorn and Pascho, 2000). As such, the presence of a bacterium can be determined from the blood; additionally, the antibody response can also be measured to determine the

degree of infection (Alcorn and Pascho, 2000). *Renibacterium salmoninarum* has been detected from the blood of brook trout, brown trout, coho salmon, rainbow trout, and sockeye salmon (Bullock and Snieszko, 1969; Pascho et al., 1987; Rhodes et al., 1998; Alcorn and Pascho, 2000), although its capability to be detect *R. salmoninarum* in a manner comparable to kidney and spleen samples has yet to be determined.

4.1.4. Mucus

As an active part of the fish immune system, the mucus is well known to be a vital barrier for various pathogens (Hjelmeland et al., 1983). It also has a protective role in fish immunity and contains proteolytic enzymes, lymphocytes, antibodies, and lysozyme, which are important components of the fish immune response (Ourth, 1980; Hjelmeland et al., 1983; St. Louis-Cormier et al., 1984; Ellis, 2001). While *R. salmoninarum* has been detected in skin lesions from rainbow trout and coho salmon, it has not been detected from solely the mucus (Hoffmann et al., 1984). However, due to its role in the immune response, it is likely that *R. salmoninarum* would be detected from the mucus layer.

4.1.5. Urine and/or feces

Though the horizontal transmission of *R. salmoninarum* is still not fully understood, it has been suggested that *R. salmoninarum* can be transmitted via the feces of infected fish (Austin and Rayment, 1985; Fryer and Lannan, 1993; Balfry et al., 1996). *Renibacterium*

salmoninarum has been detected from within the intestine of Chinook salmon and rainbow trout (Bullock et al., 1978), and from the feces of brown trout, brook trout, coho salmon, and rainbow trout (Bullock et al., 1980; Mitchum and Sherman, 1981). Additionally, Bruno (1986) demonstrated the presence of *R. salmoninarum* within the endothelial cells of the urinary tract of experimentally infected rainbow trout and Atlantic salmon, suggesting that *R. salmoninarum* could be passed to the external environment via the urinary tract.

4.2. Isolation and bacteriological identification of the agent

As discussed previously, a number of culture media have been successfully used for the primary isolation of *R. salmoninarum* from clinically infected fish. Among these media, cysteine blood agar (Ordal and Earp, 1956), KDM2 (Evelyn, 1977), SKDM (Austin et al., 1983), charcoal agar medium (Daly and Stevenson, 1985), and MKDM (Faisal et al., 2010) are used with varying degrees of success. The most common drawback of bacterial culture is the slow growing nature of *R. salmoninarum*, which can require up to 12 weeks to achieve bacterial growth. The optimal incubation temperature for the isolation of *R. salmoninarum* on culture media is 15°C and the organism is differentiated from other Gram-positive bacteria using the morpho-chemotaxonomic features described by Sanders and Fryer (1980).

4.3. Antigen-antibody reactions

Several diagnostic assays have been developed over the past 40 years to detect the presence of *R. salmoninarum*-specific antigens and antibodies, including the agglutination test, various fluorescent antibody tests, enzyme linked immunosorbent assays, and an immunohistochemistry test.

4.3.1. Agglutination test

Although easy and rapid to perform, the agglutination test requires that bacteria are first cultured, which conveys no advantage if compared with that of other diagnostic methods. To develop a coagglutination test to detect *R. salmoninarum* in kidney tissues, Kimura and Yoshimizu (1981) used *Staphylococci*-specifically sensitized with antibodies against *R. salmoninarum* with limited success.

4.3.2. Immunofluorescence

Direct and indirect fluorescent antibody tests (FAT) have commonly been used to detect *R. salmoninarum* in infected tissues, including fixed and paraffin-embedded tissues. Bullock and Stuckey (1975) were the first to describe the indirect fluorescent technique (IFAT) to visualize *R. salmoninarum* cells in tissues of infected fish. They concluded that IFAT is more sensitive than Gram staining and can detect the bacteria in subclinical infections. Several

methods to quantify *R. salmoninarum* utilizing FAT tests have been used, including a subjective scoring of fluorescence intensity (1+ to 4+) in tissue smears (Bullock et al., 1980). In a later procedure, bacteria are immobilized on filter-paper grids and titers expressed as cells per unit of tissue or ovarian fluid (Elliott and Barila, 1987).

Elliott and McKibben (1997) compared two fluorescent antibody techniques, membrane filtration FAT (MF-FAT) and smear-FAT (S-FAT) for detection of *R. salmoninarum* in ovarian fluid from naturally infected Chinook salmon. They reported greater sensitivity of MF-FAT compared to the S-FAT and concluded that MF-FAT was preferable for detection of low numbers of bacteria. Cross reactivity of other bacterial species with antisera prepared against *R. salmoninarum* has been reported (Bullock et al., 1980; Austin et al., 1985; Brown et al., 1995), thus the inclusion of control material from *R. salmoninarum*-positive fish is necessary for comparison of cell morphology and staining properties of bacteria in test and control samples (Elliott and McKibben, 1997). Inter-laboratory comparisons revealed that FAT reproducibility can be poor when used in detection of very low levels of infection (Armstrong et al., 1989).

4.3.3. Enzyme linked immunosorbent assay (ELISA)

A double antibody sandwich ELISA, also known as quantitative ELISA (Q-ELISA), can provide an indication of the real prevalence of BKD in a fish population due to its ability to determine both prevalence and intensity of the infection (Pascho et al., 1998). The procedures are fairly standardized by the studies of Pascho and Mulcahy (1987) and Pascho et al. (1991). A positive threshold can be computed for Q-ELISA results interpretation (Meyers et al., 1993;

Pascho et al., 1998). The positive-negative cutoff absorbance for the kidney homogenate was determined as 0.10, with the following antigen level categories for positive kidney samples: low (0.10 to 0.19), medium (0.20-0.99), and high (1.00 or more) (Pascho et al., 1998).

Hsu et al. (1991) developed an improved monoclonal antibody based ELISA assay for detection of the p57 protein of *R. salmoninarum*. The assay is both specific and sensitive for detection of soluble *R. salmoninarum* antigen at concentrations as low as 50-100 ng/ml.

4.3.4. Immunohistochemistry (IHC)

Immunohistochemistry has the advantage of simultaneously visualizing *R. salmoninarum* and the tissue alteration caused by the infection (Jansson et al., 1991; Evensen et al., 1994). Immunohistochemistry has been used to detect natural and experimental infections of BKD. For example, using *in situ* IHC, Lorenzen et al. (1997) reported the first detection of *R. salmoninarum* in rainbow trout in Denmark. Evensen et al. (1994) detected the organism *in situ* by using IHC in paraffin embedded tissue specimens from Atlantic salmon, using monoclonal antibodies specific for the *R. salmoninarum* p57 protein. However, it has been reported that prolonged preservation of tissue samples in formalin has deleterious effects on antigen detection and retrieval in immunohistochemical assays (Evensen et al., 1994).

4.4. Polymerase chain reaction

Polymerase chain reaction has been successfully used to detect very low rates of *R. salmoninarum* infection. For example, *R. salmoninarum* DNA was detected within individual Chinook salmon eggs with a sensitivity of 2 bacterial cells/egg (Brown et al., 1994). A nested PCR (nPCR) developed by Chase and Pascho (1998) amplifies a 320-base pair (bp) fragment of the gene encoding the p57 protein. These authors also recorded no specific fragment amplification when other fish bacterial pathogens were used for templates for the nPCR (Chase and Pascho, 1998). The sensitivity of the nested method increased one hundred-fold compared to the conventional PCR method (Pascho et al., 1998). Pascho et al. (1998) also compared the sensitivities of nPCR, ELISA, and FAT assays in the detection of *R. salmoninarum* in kidneys of infected Chinook salmon. They concluded that nPCR showed the highest sensitivity, followed by ELISA, and then FAT (Pascho et al., 1998). Pascho et al. (1998) also reported that nPCR detected *R. salmoninarum* in 100% of the tested ovarian fluid samples, concluding that nPCR was the most accurate and sensitive method for detection of *R. salmoninarum*. Hong et al. (2002) designed a pair of specific primers for nested amplification of 501 bp and 314 bp DNA fragments of the sequence coding p57 of *R. salmoninarum* and also recorded no specific fragment amplification when other principal fish bacterial pathogens were used as templates. However, Miriam et al. (1997) have cautioned that PCR positive samples may contain some proportion of dead *R. salmoninarum* with a detectable level of DNA, implying that kidney tissues containing non-culturable *R. salmoninarum* (i.e., no live bacterial cells) can be falsely positive when tested with nPCR.

In addition to the nested PCR, Halaihel et al. (2009) established a quantitative reverse transcriptase-PCR (RT-qPCR) to detect *R. salmoninarum* in kidney tissue samples. The RT-qPCR technique is able to detect low numbers of viable bacterial messenger RNA, implying a higher capacity of detecting chronically infected animals. Additionally, Chase et al. (2006) developed and assessed a quantitative polymerase chain reaction (qPCR) assay for the detection and enumeration of *R. salmoninarum*, allowing scientists to determine the level of intensity of an infection. The qPCR amplifies a 69-bp region of the gene encoding the major soluble antigen of *R. salmoninarum*, and consistently detected as few as 5 *R. salmoninarum* cells/reaction in kidney tissue (Chase et al., 2006).

5. Differential diagnosis

External manifestations of BKD are non-pathognomonic, but the course of the disease and the granulomatous nature of the kidney lesions may provide presumptive identifications. The disease can be differentiated from other kidney diseases of chronic progression including proliferative kidney disease, manifested as lymphoid hyperplasia in response to the myxozoan parasite *Tetracapsula bryosalmonae* (Clifton-Hadley et al., 1984), nephrocalcinosis, which is calcium deposits in the kidney (Peddie, 2004), and pseudo-kidney disease, caused by the bacterium *Carnobacterium piscicola* (Ross and Toth, 1974). Differentiation is mainly based on observation and detection of the organism or its antigens using immunofluorescence, IHC, ELISA, or PCR. In the case of nephrocalcinosis, differentiation is mainly based on bacteriological assessment to initially determine the presence of the bacterium; however on-farm examination

of lesion consistency can help to discriminate between these conditions as BKD lesions are soft, whilst those caused by nephrocalcinosis have a gritty texture (Peddie, 2004).

Renibacterium salmoninarum can be differentiated from other bacteria in the coryneform group, which includes the genera of *Listeria*, *Erysipelothrix*, *Corynebacterium*, *Actinomyces*, *Celullomonas*, *Curtobacterium*, *Arthrobacter*, and *Brevibacterium*, by cell wall composition and G + C contents of DNA (Stuart and Welshimer, 1974; Sanders and Fryer, 1980). In particular, even though *R. salmoninarum* shares certain characteristics with *Actinomyces pyogenes* (formerly *Corynebacterium pyogenes*), they differ in a number of other characteristics. *Actinomyces pyogenes* is facultatively anaerobic, catalase negative, and produces acid from carbohydrates (Holt et al., 2001). Also, the genus *Renibacterium* can be separated from pathogenic *Corynebacteria* and *Caseobacter* by the presence of lysine in the cell wall and the absence of mycolic acids (Crombach, 1978). *Caseobacter* is further differentiated by a mol % G + C of 60-67 and *Celullomonas* contains the diamino acid ornithine in its cell wall peptidoglycan and has a mol % G + C ranging from 65-72 (Crombach, 1978).

Interestingly, some of the coryneform groups of bacteria have overlapping characteristics and phylogenetic homology. Among this group of bacteria, a peptidoglycan cell wall containing lysine occurs primarily in the *Arthrobacter* and *Brevibacterium* genera (Holt et al., 2001). DNA homology studies also show a close relationship between several species in these two genera. However, these bacteria have usually been isolated from the environment, are chemo-organotrophic, show a progression of morphological changes during the growth cycle, and have a mol % G + C above 60 (Holt et al., 2001). Interestingly, all of these characteristics are distinctly different from that of *Renibacterium*.

6. Discrepancies among diagnostic tests

Frequently, when multiple diagnostic assays are used to determine a *R. salmoninarum* infection in the same sample, discrepancies among the results can occur, thus making diagnosis difficult (Cipriano et al., 1985; Pascho et al., 1987; Sakai et al., 1986; Griffiths et al., 1991; Gudmundsdóttir et al., 1993; Meyers et al, 1993; White et al., 1995; Faisal and Eissa, 2009; Nance et al., 2010; Elliott et al., 2013). However, as each of the methods targets different components of the bacterium, the inconsistencies are not entirely unexpected. Molecular assays like PCR are designed to detect the presence of DNA, which does not distinguish if the pathogen is alive or dead (Pascho et al., 1998). Alternatively, propagating the bacterium *in vitro* requires the presence of viable bacteria at high numbers to ensure isolation (Miriam et al., 1997). Also, commonly used serological techniques, such as ELISA, detect antigens secreted by the pathogen (Pascho et al., 1998).

Faisal and Eissa (2009) suggested that the disagreement in results among assays may reflect different phases of a *R. salmoninarum* infection at the time of sampling. In their study, these authors documented six patterns of testing results, with each of the patterns representing a potential stage along the course of a natural *R. salmoninarum* infection (initial, established, well-established, recovery, advanced recovery, and no exposure/eliminated). The authors also proposed that by studying the patterns of infection, the course of BKD infections in a particular population could be determined. Furthermore, Nance et al. (2010) also suggested using inconsistent results from diagnostic tests to infer upon the state of infection of individual fish.

7. Control Methods

7.1. Chemotherapy

Since the early 1950s, a relatively large number of chemotherapeutics have been intensively tested *in vivo* and *in vitro* for efficacy in treating BKD. Rucker et al. (1951) was first to use antimicrobial agents against clinical BKD and their results showed a definite decrease in mortalities when sulfadiazine was incorporated into fish diets. Although treatment does not completely cure clinically sick fish, sulfamerazine can reduce BKD mortalities alone and when combined with sulfaguanidine and sulfadiazine (Allison, 1958). Wolf and Dunbar (1959) tested 34 therapeutic agents, including erythromycin, thiocyanate, and sulfamerazine, on 16 strains of *R. salmoninarum* using the disk method for drug sensitivity screening, followed by *in vivo* feeding trials with experimentally infected fish. They concluded that erythromycin fed at the rate of at least 100 mg/kg of fish for 21 days gave the best results (Wolf and Dunbar, 1959). Generally, due to the occurrence of the bacterium intracellularly as well as extracellularly, these treatments only suppressed the systemic spread of the organism and induced partial relief (Amos, 1977). Intramuscular (i.m.) and i.p. administration of sulfonamide drugs significantly reduced prespawning mortality among Chinook salmon broodstocks being held prior to spawning (Amend and Fryer, 1968). However, sulfonamides administered by i.m. or i.p. routes often produced sterile abscesses at the injection site in adults, and can induce mortalities and teratogenicity within their progeny (Amos, 1977).

In an attempt to reduce or prevent vertical transmission of BKD, salmon eggs can be water hardened for one hour in two ppm erythromycin (Amos, 1977). However, in this process, erythromycin was rapidly eliminated from the eggs and dropped below detectable levels in the eggs within 24 hours after water hardening (Evelyn et al., 1986a, b). Interestingly, erythromycin remains in the eggs of injected females for up to 60 days before spawning (Evelyn et al., 1986a, b; Moffitt, 1991). It is believed that erythromycin residues inside the eggs assist in preventing vertical transmission of *R. salmoninarum* from parents to their offspring (Lee and Evelyn, 1994). Detectable amounts of erythromycin often remain in the perfused tissues of both juvenile and adult salmon long after they are no longer detected in the plasma and muscle, which possibly contributes to the efficacy of erythromycin against the slow growing *R. salmoninarum* (Moffitt, 1991; Haukenes and Moffitt, 1999). Feeding erythromycin can also efficiently reduce mortalities of infected hatchery raised salmonids, with a dose of 200 mg/kg body weight for 21 days being the most effective (Wolf and Dunbar, 1959; Austin, 1985; Moffitt and Bjornn, 1989; Moffitt, 1992). Monthly subcutaneous injections of adult female Pacific salmon with 11 mg/kg erythromycin can also result in reduced pre-spawning mortality due to BKD (Klontz, 1983). Erythromycin is only available as an Investigational New Animal Drug through the United States Food and Drug Administration (Moffitt, 1992).

Austin (1985) evaluated the efficacy of more than 70 antimicrobial compounds both *in vivo* and *in vitro* and found that penicillin G, kitasamycin, erythromycin, spiramycin, and clindamycin were useful for reducing early clinical BKD cases, while lincomycin, rifampicin, and cephradine were effective prophylactically, but had limited use therapeutically. Hsu et al. (1994) tested the efficacy of enrofloxacin in treating BKD *in vitro* and *in vivo* and they concluded

that high bioavailability, low minimal inhibition concentrations, and large volume distribution of the antibiotic make it a potential candidate for use as an effective therapeutic against BKD.

7.2. Adult segregation

Broodstock segregation is a more practical method for reducing the prevalence and levels of *R. salmoninarum* in hatchery-reared salmon (Pascho et al., 1991) and for increasing survival during their downriver migration and entry into seawater (Pascho et al., 1993; Elliott et al., 1995). This procedure aims to interrupt the vertical transmission of *R. salmoninarum* by destroying eggs from parental brood fish that exhibit clinical signs of BKD, or that test positive with a high titer against *R. salmoninarum* antigens. Along with stricter hygienic measures, Faisal et al. (2012) documented a drastic decline in BKD prevalence in Michigan hatcheries operated by the Michigan Department of Natural Resources after the implementation of broodstock screening and culling. The method is used successfully in a number of U.S. states and Canadian provinces, such as Washington, Idaho, Wisconsin, and Ontario.

7.3. Eradication

Due to the complicated nature of BKD and its threats to fisheries, Hoskins et al. (1976) recommended complete destruction of infected stocks and disinfection of the holding facilities to achieve complete eradication of the disease. However, due to the widespread occurrence of

R. salmoninarum, this procedure is considered by fisheries managers to be impractical (Sanders and Fryer, 1980).

Eradication can still be of value in single fish farms or hatcheries that receive their water supply from a specific pathogen-free source (European Commission, 1999). Eradication procedures should be followed by standard cleaning and disinfection procedures. Although some trials have been attempted to eradicate BKD from fish farmed in open waters (e.g., sea and lake cages) or from farms and hatcheries with water supplies from rivers, results were very discouraging (European Commission, 1999).

After eradication procedures have been applied in the fish farms and hatcheries, restocking should only utilize certified BKD-free stocks. Restocking should be followed by two inspections and laboratory examinations per year for a total period of two years before the facility can be designated as "BKD-free" (European Commission, 1999).

7.4. Prophylaxis

7.4.1. Reducing the risk of BKD introduction

Special attention should be paid to prevent the introduction of infected fish or their gametes (Evelyn et al., 1984; Yoshimizu, 1996). This can only be achieved through prior examination and quarantine. Specific efforts should be made to restrict the movement of vehicles associated with the facility, restrict visitors to the facility, and to utilize separate nets, buckets, and brushes for each raceway and building associated with the facility. Repopulation

must be accompanied with certification by a competent authority declaring that the fish or eggs are specific pathogen free.

7.4.2. Vaccination

In the last three decades, vaccination against BKD has achieved different levels of success. Paterson et al. (1981) reported that an inactivated suspension of *R. salmoninarum* mixed 1:1 with Freund's adjuvant administered by i.p. injection reduced the level of infection of *R. salmoninarum* in yearling salmon, but did not completely eliminate the infection. McCarthy et al. (1984) examined the use of a pH-lysed bacterin in rainbow trout, delivered via immersion and i.p. injection, and while it provided some protective immunity, the authors acknowledged that more detailed diagnostic testing techniques needed to be used to more accurately ensure that vaccinated fish were free from infection. Sakai et al. (1993) found that although vaccination evoked specific antibodies, these antibodies did not endow fish with any significant protection. Wood and Kaattari (1996) evaluated a formalin-killed *R. salmoninarum* vaccine that contained the p57 protein (p57+), and a vaccine which lacked most of the p57 protein (p57-), in Chinook salmon. These authors were able to determine that the p57- vaccine produced antibody titers five times higher than the p57+ vaccine, demonstrating an enhanced antibody response. Piganelli et al. (1999) demonstrated that oral administration of *R. salmoninarum* expressing low levels of cell associated p57, resulted in an extension of the mean time to death after challenge and they concluded that the protection was not due to humoral antibodies. This conclusion supported earlier histopathological indications of an involvement of the cell

mediated immune response in recovery, due to intracellular survival and the composition of inflammatory cells in connection with signs of regression (Munro and Bruno, 1988). Rhodes et al. (2004) presented DNA adjuvants and whole bacterial cell vaccines against *R. salmoninarum* that were tested in Chinook salmon fingerlings. These authors concluded that whole cell vaccines of either a nonpathogenic *Arthrobacter* spp. or an attenuated *R. salmoninarum* strain produced limited protection against acute intraperitoneal challenge with virulent *R. salmoninarum*. They also concluded that the addition of either synthetic oligodeoxynucleotides or purified *R. salmoninarum* genomic DNA as adjuvants did not increase protection; however, a combination of both whole cell vaccines significantly increased survival among fish naturally infected with *R. salmoninarum*. Also, the surviving fish treated with the combination vaccine exhibited reduced levels of bacterial antigens in the kidney. There have also been several other studies examining the use of the nonpathogenic *Arthrobacter* spp. vaccine (known commercially as Renogen®), all with varying and inconsistent results (Burnley et al., 2010; Alcorn et al., 2005; Salonius et al., 2005).

APPENDIX

Table 1.1. Summary of the morphological and biochemical characteristics of *Renibacterium salmoninarum* (Eissa, 2005; Austin and Austin, 2007). *API-ZYM is a bacterial enzyme based assay used for the specific identification of different bacteria.

Test	Criteria	Notes
Agar hydrolysis	-	
Amylase	-	
Arginine hydrolysis	-	
Bile solubility	-	
Butyrate esterase	-	
Caprylate esterase	-	
Carbohydrate utilization	-	
Casein hydrolysis	+	
Catalase	+	
Cytochrome oxidase	-	
DNase	+	(-) By API-ZYM*
Esculin hydrolysis	-	
Gelatin liquefaction	+	(-) By API-ZYM*
Gram stain	+	
Hemolytic activity	β-hemolytic	Complete clearance zone around bacteria
Indole test	-	
Methyl red	-	
Nitrate reduction	-	
Oxidase	-	
PAS (Periodic Acid Schiff) stain	+	
Phosphatase	-	
Trypsin	+	
Tween-20, 40, and 60 hydrolysis	+	
Tween-80 hydrolysis	-	
Urease	-	
Zeihl-Nielsen (Acid Fast) stain	-	Non-acid fast

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Chapter 2

Epidemiological investigation of *Renibacterium salmoninarum* in three *Oncorhynchus* spp. in Michigan from 2001 to 2010

Faisal, M., Schulz, C., Eissa, A., Brenden, T., Winters, A., Whelan, G., Wolgamood, M., Eisch, E., and VanAmberg, J. 2012. Epidemiological investigation of *Renibacterium salmoninarum* in three *Oncorhynchus* spp. in Michigan from 2001 to 2010. Prev. Vet. Med. 107: 260-274.

1. Abstract

Bacterial kidney disease (BKD) has caused mortalities and chronic infections in wild and farm-raised salmonids throughout the world. In the Laurentian Great Lakes of North America, BKD was associated with several large-scale mortality events of *Oncorhynchus* spp. throughout the 1980s and 1990s. In response to these mortality events, the state of Michigan implemented several enhanced biosecurity measures to limit the occurrence of BKD in state-operated hatcheries and gamete-collection weirs. The objectives of this study were to assess if infection levels (prevalence and intensity) of *Renibacterium* salmoninarum, the causative agent of BKD, have changed in broodstock and pre-stocking fingerlings of three feral *Oncorhynchus* spp. [Chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), and steelhead (*O. mykiss*)] over a decade, following the implementation of the enhanced biosecurity measures. Between 2001 and 2010, a total of 3,530 broodstock salmonids collected from lakes Huron and Michigan tributaries during spawning runs and 4,294 propagated pre-stocking salmonid fingerlings collected from three state of Michigan fish hatcheries were tested for the presence of *R. salmoninarum* antigens using the enzyme-linked immunosorbent assay. Substantial declines in the overall prevalence of the bacterium were detected in each of the examined broodstocks. Most propagated pre-stocking fingerlings also exhibited substantial declines in *R. salmoninarum* prevalence. Prevalence was typically higher in Chinook salmon from Lake Michigan than from Lake Huron; prevalence was also generally higher in the Hinchenbrooke strain of coho salmon than in the Michigan-adapted strain. For most strains and stocks examined, intensity of *R. salmoninarum* infection was found to have declined. Although there were declines in the

potential for shedding the bacteria for both male and female Chinook and coho salmon, overall shedding rates were generally low (<15%) except for Hinchenbrooke coho salmon strain, which had shedding prevalences in excess of 50% at the beginning of the study. This study provides evidence that enhanced biosecurity measures at culture facilities and collection sites are capable of severely curtailing disease infection in wild populations even at the scale of Lake Michigan fisheries.

2. Introduction

The Laurentian Great Lakes (LGL) support a diverse fish community, including Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), and steelhead (*O. mykiss*; the migratory strain of rainbow trout), which are both recreationally and ecologically important. Even though these three species have supported valuable sport fisheries for a number of decades, they are not native to the LGL and were initially introduced to exert predatory pressure on and reduce densities of non-native pelagic prey fishes (mainly alewives, *Alosa pseudoharengus*), and further expand LGL sportfishing opportunities (Keller et al., 1990; Holey et al., 1998; Hansen and Holey, 2002). The stocking attempts proved to be successful and high quality recreational fisheries quickly became established (Hansen and Holey, 2002; Tanner and Tody, 2002).

The successful introduction of Chinook salmon, coho salmon, and steelhead to the LGL and the popular sport fisheries that resulted, led the State of Michigan to develop a Pacific salmonid rearing program in several state-operated fish hatcheries (Dexter and O'Neal, 2004).

Chinook salmon, coho salmon, and steelhead are propagated annually using egg and milt samples collected at gamete-collection weirs operated by the Michigan Department of Natural Resources (MDNR) on several LGL tributaries (Dexter and O'Neal, 2004). Fish are raised in the hatcheries until they are between 6 and 18 months of age (depending on the species) and are stocked at several locations in the LGL, as well as in a number of LGL streams in Michigan (Dexter and O'Neal, 2004).

Among the pathogens that *Oncorhynchus* spp. are susceptible to is the Gram-positive diplobacillus *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), which is transmitted both horizontally and vertically and hence extremely difficult to control. BKD can take the form of chronic and acute infections and is characterized by the formation of granulomas in kidneys and other visceral organs (Fryer and Sanders, 1981). The disease was first documented in Michigan hatcheries in 1955 (Allison, 1958) and has since spread to all of the Great Lakes, as well as several inland lakes and rivers in the LGL region (Allison, 1958; MacLean and Yoder, 1970; Holey et al., 1998; Beyerle and Hnath, 2002; Eissa et al., 2006; Nuhfer, 2006; Faisal et al., 2010).

BKD has been associated with several mortality events of *Oncorhynchus* spp. in the LGL (MacLean and Yoder, 1970; Holey et al., 1998). MacLean and Yoder (1970) documented the presence of *R. salmoninarum* in dead coho salmon from lakes Michigan and Superior. During the period from 1988 to 1992, annual mortality events of Chinook salmon in Lake Michigan occurred, which was attributed at least in part to a BKD epidemic (Holey et al., 1998). These die-offs had drastic effects on the Lake Michigan Chinook salmon fishery, with the recreational

fishery yield declining approximately four-fold between the mid and late 1980s (Hansen and Holey, 2002).

In response to the BKD epidemic, the MDNR initiated a number of enhanced biosecurity practices at state-operated hatcheries and gamete-collection weirs to limit the occurrence and spread of BKD (Table 2.1). The MDNR expanded its biosecurity practices to include clinical inspections, culling, egg disinfection, hardening the eggs in water containing the antibiotic erythromycin, regular screening of propagated fish, and treatment with antibiotics, such as erythromycin. While erythromycin is not yet fully approved by the U.S. Food and Drug Administration to treat BKD, limited use of the antibiotic is allowed as an investigational new animal drug exemption.

The objectives of this study were to assess if infection levels (prevalence and intensity) of *R. salmoninarum* in broodstock and pre-stocking fingerlings from three feral *Oncorhynchus* spp. during the decade 2001–2010, have changed with the enhanced biosecurity practices. An additional objective of the study was to assess the role of shedding *R. salmoninarum* in the gametes of the broodstock on the overall prevalence and intensity of *R. salmoninarum* in pre-stocking fingerlings.

3. Materials and Methods

3.1. Fish collection

Between 2001 and 2010, a total of 3,530 feral, spawning Chinook and coho salmon and steelhead were collected from MDNR gamete-collection weirs in Michigan, USA (Figure 2.1; Table 2.2). Ages of the fish ranged from approximately 3–5 years. For this study, the five species/stocks/strains of fish were designated based on the location of collection (or source of gametes) and fish species or strain: Chinook salmon from the Little Manistee River Weir (LMRW-CHS), Chinook salmon from the Swan River Weir (SRW-CHS), the Hinchenbrooke strain of coho salmon from the Platte River Weir (HB-COS), the Michigan-adapted strain of coho salmon from the Platte River Weir (MI-COS), and steelhead from the Little Manistee River Weir (LMRW-STT; Table 2.2). Chinook and coho salmon were sampled during the months of September and October of every year, while steelhead were sampled in April of every year. Overall, male and female fish were collected in roughly equal proportions, although sex ratios did vary across the sampling periods.

In addition to the broodstock, 4,294 propagated pre-stocking fingerlings were evaluated for *R. salmoninarum* (Table 2.3). Fish were between 6 and 18 months of age at time of sampling. Chinook salmon fingerlings were propagated at the Platte River State Fish Hatchery (PRSFH), the Thompson State Fish Hatchery (TSFH), and the Wolf Lake State Fish Hatchery (WLSFH). However, prevalence declines were not assessed for LMRW-CHS at the TSFH because there was only one year of data collection. Coho salmon fingerlings were only propagated at

the PRSFH. Steelhead fingerlings were propagated at the TSFH and WLSFH (Figure 2.1). The environmental conditions at the hatcheries (water temperature, dissolved oxygen levels, fish densities, etc.) were monitored and remained optimal for fish rearing, while reducing the risk of disease.

The number of fish sampled varied depending on the availability of fish returning to spawn at the gamete-collection weirs and the number of fish available from MDNR hatcheries (Tables 2.2 and 2.3). Additionally, sacrificing large groups of fish (i.e., at least 60 fish) is required to provide a 95% confidence of detecting the pathogen, with an assumed minimum incidence of 5% of the disease (Hnath, 1993; Ossiander and Wedemeyer, 1973). A description of the enhanced biosecurity measures implemented by the MDNR at gamete-collecting weirs and hatchery facilities is provided in Table 2.1.

3.2. Sample collection

3.2.1. Broodstock

Length, weight, and sex of all fish collected by MDNR personnel at gamete collection weirs were recorded. Each fish was also thoroughly examined both externally and internally for clinical signs of BKD or other diseases. The gross pathology that was observed included exophthalmia, ascites, granulomas in the kidneys, and swelling and congestion of hematopoietic organs. Samples (<5 g) of kidneys and spleens were removed in the field and stored on ice in whirlpaks (VWR International, West Chester, PA), as recommended by the

American Fisheries Society Bluebook (2012) and the World Organization for Animal Health (OIE, 2012), and frozen at -20°C until processing. Additionally, approximately 5 ml of ovarian fluid or milt were collected from the same feral fish, stored on ice in 15 ml centrifuge tubes (Denville Scientific, Inc., Metuchen, NJ), and frozen at -20°C once returned to the laboratory. Samples were frozen at -20°C for no longer than 30 days before being processed by laboratory personnel.

3.2.2. Pre-stocking fingerlings

Propagated fish were collected within the hatcheries and euthanized with an overdose (250 mg/L) of Tricaine Methanesulfonate (MS-222, Argent Chemicals, Redmond, WA). Lengths and weights of each fish were recorded. Each fingerling was examined both externally and internally for clinical signs of BKD infection. The gross pathology that was observed included exophthalmia, ascites, granulomas in the kidneys, and swelling and congestion of hematopoietic organs. The entire kidney and a sample of spleen were aseptically removed, stored in whirlpaks, and frozen at -20°C until processing. Samples were frozen at -20°C for no longer than 30 days before being processed by laboratory personnel.

3.3. Sample processing

Kidney and spleen tissue samples were diluted 1:4 (w:v) with Hank's Balanced Salt Solution (HBSS, Sigma–Aldrich, St. Louis, MO) and homogenized on high speed for 2 min with a

Biomaster Stomacher (Wolf Laboratories Limited, Pocklington, York, UK) as described by Faisal et al. (2009). Gamete samples were also diluted 1:4 (w:v) with HBSS and vortexed on high speed for approximately 30 s. Each homogenized kidney and spleen tissue sample and gamete sample were then tested for the presence of *R. salmoninarum* using the quantitative enzyme-linked immunosorbent assay (Q-ELISA).

3.4. Q-ELISA

The general Q-ELISA protocol outlined in Pascho and Mulcahy (1987), with modifications recommended by Gudmundsdóttir et al. (1993) and Olea et al. (1993), was used to assess *R. salmoninarum* antigens in all sampled fishes. Homogenized spleen, kidney, and gamete samples (250 µl) were aliquoted into 1.5-ml microcentrifuge tubes (DOT Scientific, Inc., Burton, MI) containing 250 µl of Phosphate Buffered Saline with Tween-20 (PBS-T20; Sigma) and 5% goat serum (Sigma) and 50 µl of CitriSolv (Fisher Scientific, Pittsburgh, PA). The purpose of the CitriSolv solvent was to dissolve and remove liquids from the aqueous supernatant (Gudmundsdóttir et al., 1993), while the introduction of 5% goat serum was to increase the sensitivity of the assay (Olea et al., 1993). Additionally, Pascho and Mulcahy (1987) demonstrated the excellent specificity of the procedure by showing that the Q-ELISA did not react with antigens from 11 species of bacteria, including the common fish pathogens *Aeromonas salmonicida*, *Vibrio anguillarum*, and *Yersinia ruckeri*. Samples were vortexed for approximately 10 s, heated at 100°C for 15 min, and then centrifuged at 14,000 rpm for 10 min. The aqueous supernatant of each sample was used for Q-ELISA testing. The positive–negative

cut-off absorbance for the samples was 0.10 (Meyers et al., 1993). Samples that tested positive were assigned the following antigen levels: low (0.10–0.199), medium (0.20–0.999), and high (≥1.00), as recommended by Meyers et al. (1993) and Pascho et al. (1998). Each assay included two negative controls (a negative fish kidney and spleen sample and a dilution buffer) and two positive controls (a positive kidney and spleen sample and the standards supplied with the kit).

Broodstock specimens were separated into one of four infection categories based upon Q-ELISA results from the kidney/spleen and gamete samples: (1) individuals that were negative for *R. salmoninarum* in the kidney and spleen sample and the gamete sample (KS−/G−); (2) individuals that were positive for *R. salmoninarum* in the kidney and spleen sample and negative in the gamete sample (KS+/G−); (3) individuals that were negative for *R. salmoninarum* in the kidney and spleen sample and positive in the gamete sample (KS−/G+); and (4) individuals that were positive for *R. salmoninarum* in the kidney and spleen sample and the gamete sample (KS+/G+). Individuals that were positive for *R. salmoninarum* in the gamete sample were considered to be potential shedders of the bacterium.

3.5. Data analyses

Logistic regression was used to evaluate how *R. salmoninarum* prevalence in broodstock and propagated pre-stocking fingerlings had changed over time. Logistic regression was also used to evaluate how prevalence of broodstock shedding had changed over time. For each response variable, a series of models that differed with respect to model intercepts and slopes were fit to observed infection or shedder data. A complete listing and description of the

models is presented in Table 2.4. For assessing *R. salmoninarum* prevalence in broodstock, the most complex model had species/stock/strain-specific intercepts and slopes. For assessing *R. salmoninarum* prevalence in pre-stocking fingerlings, the most complex model had species/stock/strain/hatchery-specific intercepts and slopes. For assessing shedding prevalence in broodstock, the most complex model had species/stock/strain/sex-specific intercepts and slopes. For each fitted model, Akaike information criterion (AIC) was calculated to evaluate model goodness of fit (a low AIC value indicates a parsimonious model that provides a good fit to the data; Burnham and Anderson, 2002).

Changes in rates of *R. salmoninarum* infection intensities over time for both broodstock and propagated pre-stocking fingerlings were evaluated using multinomial logistic regression. Multinomial logistic regression was also used to assess changes in the different shedding categories (i.e., gamete− and gamete+) over time. We used multinomial logistic regression models rather than cumulative logit models because generally the data did not meet the proportional odds assumption necessary for a cumulative logit model (Agresti, 2007). When fitting the multinomial logistic regression models to infection intensities, we used the negative infection category (Q-ELISA < 0.10) as the reference category. When fitting the multinomial logistic regression models to the shedder categories, we used KS−/G− as the reference category. Thus, the multinomial regression models for the infection intensity levels fit models that evaluated how the log-odds of the low, medium, and high infection intensity levels changed relative to that of non-infected category. For the shedding data, the multinomial logistic regression models evaluated how the log-odds of the KS+/G−, KS−/G+, KS+/G+ shedding categories changed relative to the KS−/G− shedding category. Unlike the model selection

process that was used when fitting the *R. salmoninarum* prevalence data, intensity and shedding category models were fit individually to each species/stock/strain (broodstock intensity), species/stock/strain/hatchery (propagated pre-stocking fingerling intensity), and species/stock/strain/sex (broodstock shedding) combination to simplify analysis and facilitate interpretation. All logistic and multinomial logistic regression models were fit in SAS using PROC GLIMMIX (SAS Institute Inc., 2010).

To determine if there was any association in *R. salmoninarum* prevalence between broodstock and progeny, we conducted Pearson correlation analyses on the calculated broodstock and pre-stocking fingerling prevalences. Correlation analyses were only conducted on Chinook salmon and coho salmon as there was insufficient data for steel-head for this analysis. For Chinook salmon, there was a 1 year time lag between the broodstock and pre-stocking fingerling comparisons as this species is reared as spring fingerling then released. Thus, Chinook salmon broodstock prevalence in 2007 was compared to pre-stocking fingerling prevalence in 2008. For Coho salmon, there was a 2-year time lag as fish of this species as this species is reared as yearlings then released. Thus, coho salmon brood stock prevalence in 2007 was compared to pre-stocking fingerling prevalence in 2009. Correlation analyses were conducted in SAS using PROC CORR (SAS Institute Inc., 2010).

4. Results

4.1. *Renibacterium salmoninarum* infection prevalence and intensity in salmon broodstocks

When data were combined for the five stocks, the overall prevalence of *R. salmoninarum* was 22.1% (SE = 0.7%). Coho salmon had the greatest overall prevalence of *R. salmoninarum* at 34.0% (SE = 1.3%), followed by Chinook salmon at 18.0% (SE = 0.9%) and steelhead at 3.8% (SE = 0.9%). For individual stocks, HB-COS had the greatest overall prevalence of *R. salmoninarum* at 48.2% (SE = 2.3%), followed by LMRW-CHS at 27.3% (SE = 1.6%), MI-COS at 26.2% (SE = 1.5%), SRW-CHS at 11.7% (SE = 0.9%), and LMRW-STT at 3.8% (SE = 0.9%).

The decline of *R. salmoninarum* prevalence in all five stocks was supported by the calculated AIC values, which showed that the models with the poorest fit were those that assumed prevalences had remained constant over time. These models had much greater AIC values than models that allowed prevalence to change over time. For all fitted models where infection was assumed to change over time, the estimated slope parameters describing the linear (on a logit scale) change in infection prevalence per year ranged from -0.678 to -0.901. In all cases, these slopes were significantly different from 0 at P-values less than 0.0001, providing strong indication that *R. salmoninarum* rate of infection had indeed declined over time. The four best-performing models had nearly equal AIC values, suggesting that each of these models would be almost equally useful for describing declines of *R. salmoninarum* infection data. The model with the lowest AIC value had species/stock/strain-specific intercepts and species-

specific slopes, but it performed only slightly better than the model with species-specific intercepts and species/stock/strain-specific slopes. The next best performing model had species/stock/strain-specific intercepts and a common slope, but it again performed only slightly better than the model with a common intercept and species/stock/strain-specific slopes. Additionally, the model with species/stock/strain-specific intercepts and slopes had an AIC difference of within 3, suggesting that there was at least some support for this model based on the observed data.

Because there were several models with at least some support for being the best model based on observed *R. salmoninarum* prevalence data, we chose to use AIC model averaging based on AIC weights to calculate a weighted-average of model parameters (intercepts and slopes) from those models. Only parameter estimates from those models with AIC differences of 3.0 or smaller were included in the model averaging (Burnham and Anderson, 2002). The model-averaged logistic regression slopes that were calculated for each of the species/stocks/strain combinations were -0.718 (SE = 0.055) for LMRW-CHS, -0.719 (SE = 0.058) for SRW-CHS, -0.804 (SE = 0.063) for HB-COS, -0.796 (SE = 0.058) for MI-COS, and -0.775 (SE = 0.141) for LMRW-STT. Based on these model-averaged slopes, *R. salmoninarum* prevalence was predicted to have declined at a rate of approximately 51% per year for LMRW-CHS (Figure 2.2A) and SRW-CHS (Figure 2.2B). The predicted prevalence of *R. salmoninarum* infection declined at a rate of approximately 55% per year for HB-COS (Figure 2.2C) and MI-COS (Figure 2.2D). Lastly, the *R. salmoninarum* predicted prevalence declined at a rate of approximately 54% per year for LMRW-STT (Figure 2.2E). Regardless of sampling year, HB-COS had the greatest predicted *R. salmoninarum* prevalence of the species/stocks, strains, followed by

LMRW-CHS, and MI-COS. SRW-CHS and LMRW-STT had approximately equal *R. salmoninarum* prevalences during those years where prevalence data were available for both stocks (Figure 2.2A-E).

Infection rates of the different intensity levels generally decreased for all species relative to non-infected fish (Figure 2.2F-J), the only exceptions being medium intensity infection rate for SRW-CHS (Figure 2.2G) and medium and high infection rates for LMRW-STT (Figure 2.2J). The multinomial log-odds for having a low, medium, or high infection intensity level versus not being infected declined by 3.14-10.80 log-odds units per year depending on the stock and intensity level. In the case of LMRW-CHS, predicted low, medium, and high intensity infection prevalences declined to less than 0.1% during the sampling period (Figure 2.2F). For SRW-CHS, the predicted low and high intensity infection prevalences also declined to less than 0.1% during this same time period (Figure 2.2G). There were also declines in the predicted low, medium, and high intensity infection prevalences for HB-COS (Figure 2.2H). For MI-COS, the predicted low and medium intensity infection rates declined to less than 1% in 2010 (Figure 2.2I), while the high intensity infection prevalences for MI-COS initially increased 2003; however, since then, the high infection prevalences have declined to a rate of 1.2% in 2010 (Figure 2.2I). For LMRW-STT, low intensity infection prevalences also declined throughout the study period (Figure 2.2J).

4.2. *Renibacterium salmoninarum* infection prevalence and intensity in propagated pre-stocking salmon fingerlings

When data were combined for the five stocks over the entire study period, the overall prevalence of *R. salmoninarum* was 15.0% (SE = 0.5%). For individual species, coho salmon had the greatest overall prevalence of *R. salmoninarum* at 20.0% (SE = 1.3%), followed by Chinook salmon at 18.1% (SE = 0.8%) and steelhead at 6.7% (SE = 0.7%). For individual stocks, HB-COS had the greatest overall prevalence of *R. salmoninarum* at 28.1% (SE = 2.4%), followed by LMRW-CHS at 24.2% (SE = 1.3%), MI-COS at 14.6% (SE = 1.5%), SRW-CHS at 11.4% (SE = 1.0%), and LMRW-STT at 6.7% (SE = 0.7%). When categorized by hatchery, LMRW-CHS WLSFH had an overall prevalence of 32.4% (SE = 2.1%), whereas LMRW-CHS PRSFH had an overall prevalence of 19.4% (SE = 1.7%) and LMRW-CHS TSFH had an overall prevalence of 0% (SE = 0.0%). Conversely, SRW-CHS WLSFH had an overall prevalence of 13.3% (SE = 2.2%), whereas SRW-CHS PRSFH had an overall prevalence of 18.3% (SE = 2.1%) and SRW-CHS TSFH had an overall prevalence of 4.0% (SE = 0.9%). Steelhead propagated at the TSFH and WLSFH had overall prevalences of 7.9% (SE = 0.01%) and 5.8% (SE = 0.01%), respectively.

Like the broodstock analysis, based on calculated AIC values, the models with the poorest fit to observed *R. salmoninarum* infection data for the propagated pre-stocking fingerlings were those that assumed infection rates had remained constant over time. Unlike the results from the broodstock analysis, however, for the propagated pre-stocking fingerlings there was a single model that was picked by the AIC model selection criteria as having vastly superior performance compared to the other models. The model with the lowest AIC value had

species/ stock/strain/hatchery specific model intercepts and slopes. Therefore, the best performing model demonstrated that there was a considerable difference among the species and strains of fish, as well as the hatcheries where the fish were propagated. The next best performing model had stocks/hatchery specific intercepts and species/stock specific slopes, but this AIC difference for this model was greater than 90, indicating that there was very little empirical support for this model (Burnham and Anderson, 2002).

Analysis demonstrated that the *R. salmoninarum* infection rate in propagated fish had indeed declined over time, since for most of the stock/hatchery combinations, the logistic regression slopes were negative and were significantly different from 0 at P-values < 0.0001 (Figure 2.3A-E). The only exceptions to this were for LMRW-CHS from the TSFH for which a slope could not be calculated because there was only 1 year of data collected from this hatchery, and for SRW-CHS from the TSFH which had a positive slope indicating that *R. salmoninarum* infection was increasing over time (Figure 2.3E). For the other stock/hatchery combinations, estimated slopes equaled -1.295 (SE = 0.135) for LMRW-CHS PRSFH, -1.241 (SE = 0.116) for LMRW-CHS WLSFH, -1.325 (SE = 0.176) for SRW-CHS PRSFH, -1.196 (SE = 0.252) for SRW-CHS WLSFH, -0.745 (SE = 0.111) for HB-COS PSFH, -0.730 (SE = 0.096) for MI-COS PRSFH, -2.079 (SE = 0.000) for LMRW-STT WLSFH, and -0.317 (SE = 0.0967) for LMRW-STT TSFH.

Based on these estimated slopes, *R. salmoninarum* prevalences in propagated pre-stocking fingerlings were predicted to decline by between 52% to 87% per year for each of these species/stock/strain/hatchery combinations. In terms of predicted *R. salmoninarum* prevalences, prevalence of *R. salmoninarum* for LMRW-CHS from the WLSFH was predicted to have declined to less than 0.1% in 2010 (Figure 2.3A). LMRW-CHS from the PRSFH also

experienced a major decline in *R. salmoninarum* prevalence (Figure 2.3B). For SRW-CHS from the WLSFH, prevalences were predicted to have declined to 1.4% (Figure 2.3C), whereas the predicted prevalence of *R. salmoninarum* in SRW-CHS from the PRSFH declined to less than 0.1% (Figure 2.3D).

In terms of infection intensity, there were significant declines in rates of infection at all intensity levels using non-infected as the baseline for LMRW-CHS at both PRSFH and WLSFH (Figure 2.3F-J). For LMRW-CHS at the WLFSH, predicted rates of infection at low and medium intensity levels initially increased from 2002 to 2003; however, since 2003, the predicted prevalence of low and medium intensity infection levels has declined to less than 0.1% (Figure 2.3F). Predicted prevalence of high intensity infection levels for LMRW-CHS at the WLSFH declined overall from 2002 to 2010 (Figure 2.3F). The predicted rates of infection at low, medium, and high intensities for LMRW-CHS from the PRSFH declined to less than 0.1% (Figure 2.3G). For SRW-CHS, significant declines in rates of infection for low intensity levels were detected at the WLSFH (Figure 2.3H), as well as for low and medium intensity levels at the PRSFH (Figure 2.3I). Additionally, the predicted prevalence for medium intensity levels of infection for SRW-CHS at the PRSFH declined to less than 0.1% (Figure 2.3I). For SRW-CHS from the TSFH, there was a significant increase in prevalence at medium intensity levels from 2002 to 2010 (Figure 2.3J).

Coho salmon fingerlings propagated at the PRSFH also saw a substantial decline in the prevalence of *R. salmoninarum* infections from 2003 to 2010. For HB-COS and MI-COS from the PRSFH, prevalence was predicted to have declined throughout the study period (Figure 2.4A and 2.4B). In regards to infection intensity for coho salmon, significant decreases in low rates

of infection were found for both Hinchenbrooke and Michigan-adapted strains (Figure 2.4C and 2.4D). Also, a significant decrease in medium rate of infection was found for the MI-COS (Figure 2.4D).

Moreover, the prevalence infection rates of *R. salmoninarum* in steelhead pre-stocking fingerlings also decreased considerably from 2005 to 2010. For LMRW-STT from the WLSFH, predicted prevalence declined to less than 0.1% in 2010 (Figure 2.5A); while for LMRW-STT from the TSFH the predicted prevalence declined to 4.0% in 2010 (Figure 2.5B). For LMRW-STT, significant decreases in prevalence at low intensity levels were observed for both WLSFH and TSFH (Figure 2.5C and 2.5D).

4.3. *Renibacterium salmoninarum* in gametes of spawning broodstock

Across all species/stocks/strains throughout the study period, the overall prevalence of broodstock that had *R. salmoninarum* antigens in ovarian fluid and milt was 6.2% (SE = 0.5%). By species, the overall prevalence of brood-stock with positive gametes was 11.1% (SE = 1.1%) for coho salmon, 4.3% (SE = 0.6%) for Chinook salmon, and 0.7% (SE = 0.5%) for steelhead. By species/stock/strain, the over-all prevalence of gamete positive broodstock was 3.6% (SE = 0.8%) for LMRW-CHS, 4.9% (SE = 0.8%) for SRW-CHS, 23.6% (SE = 2.4%) for HB-COS, and 4.3% (SE = 0.9%) for MI-COS. When calculated by sex, the overall prevalence of broodstock that were shedding the bacteria in gametes was 6.5% (SE = 0.7%) for females and 5.9% (SE = 0.7%) for males.

Based on the calculated AIC values, there were two models that had some support based on observed shedding data. The model with the lowest AIC value had species/stock/strain/sex-specific intercepts and slopes. The next best performing model had species/stock/strain/sex-specific model intercepts and species/stock/strain-specific slopes. As with the broodstock prevalence analysis, we used model averaging based on AIC weights to average the parameter estimates from these two models. The calculated model-averaged slopes equaled -0.515 (SE = 0.236) for female LMRW-CHS, -0.983 (SE = 0.237) for male LMRW-CHS, -0.384 (SE = 0.208) for female SRW-CHS, -0.093 (SE = 0.179) for male SRW-CHS, -2.165 (SE = 0.001) for female HB-COS, -2.132 (SE = 0.025) for male HB-COS, -0.379 (SE = 0.123) for female MI-COS, -0.430 (SE = 0.213) for male MI-COS, 0.088 (SE = 0.113) for female LMRW-STT, and 0.210 (SE = 0.018) for male LMRW-STT. Based on model-averaged slopes, gamete shedding prevalences were predicted to have declined by between 40% and 63% per year for LMRW-CHS (Figure 2.6A) and 9% and 32% per year for SRW-CHS (Figure 2.6B). Additionally, the predicted decline in gamete shedding prevalence for HB-COS was approximately 88% per year (Figure 2.6C) and between 32% and 35% per year for MI-COS (Figure 2.6D). For LMRW-STT, shedding prevalence was predicted to have increased by between 9% and 23% (Figure 2.6E).

In terms of predicted gamete shedding prevalences, for most species/stocks/strains shedding prevalence in gametes was generally less than 15% throughout the course of the study. The one exception to this was HB-COS where both females and males had gamete shedding prevalences of between 50% and 60% at the beginning of this study. However, by 2007, gamete shedding prevalences of both sexes had declined to less than 1% (Figure 2.6C). Although there were clear differences between sexes in predicted shedding prevalences for

some of the examined broodstock, results were inconsistent as to whether males or females had higher shedding prevalences. In terms of the different shedding categories, significant declines in shedding categories when compared to the KS−/G− shedding category were detected for some of the species/stock/strain/sex combinations. For HB-COS, significant declines in the KS+/G−, KS+/G+, and KS−/G+ categories were found for both sexes. For female LMRW-CHS, SRW-CHS, and MI-COS, significant declines in the KS+/G− and KS+/G+ categories were detected, while for male LMRW-CHS, SRW-CHS, and MI-COS, only significant declines in the KS+/G− category were detected. For LMRW-STT, no significant declines in shedding rates for any of the shedding categories were detected. The largest predicted changes in shedding category prevalences were in the KS+/G− category for female and male LMRW-CHS, which were predicted to have declined from between 40% and 60% in 2004 to less than 3% by 2007 (Figure 2.6A). The other large predicted change in shedding prevalences were in the KS+/G+ category for female and male HB-COS, which were predicted to have declined from between 40% and 55% in 2004 to less than 5% in 2005 (Figure 2.6C).

4.4. Relationship between *R. salmoninarum* prevalence in broodstock and progeny

Based on the correlation analyses conducted, no significant association between broodstock and progeny prevalences in Chinook salmon ($r = 0.220$; P-value = 0.517) was detected. There was, however, a statistically significant positive association found between broodstock and progeny prevalences in coho salmon ($r = 0.890$; P-value = 0.0073). A strong

positive, albeit not statistically significant, association was also found between broodstock and progeny prevalences in steelhead (r = 0.697; P-value = 0.0549).

5. Discussion

The findings of this study demonstrate that the intensity and prevalence of *R. salmoninarum* infections have decreased during the course of the last decade; not only in the feral broodstock, but also in hatchery settings. This correlated with the implementation of enhanced biosecurity practices in the state fish hatcheries. In this study, we opted to use the Q-ELISA as the only diagnostic tool to assess *R. salmoninarum* presence in fish tissues. In a previous study, it was demonstrated that Q-ELISA values were commensurate with disease progression in feral stocks (Faisal and Eissa, 2009).

There are several likely reasons why *R. salmoninarum* has rapidly declined in an area where it has been endemic for over half a century. As fish are exposed to *R. salmoninarum*, it is possible that the overall population may have shifted to individuals with heightened resistance to the pathogen (Purcell et al., 2008). Earlier studies on coho salmon have shown that resistance to BKD is linked to the transferrin gene (Suzumoto et al., 1977; Winter et al., 1980). The authors reported that coho salmon with the transferrin genotype of 'AA' are three times more likely to die from a *R. salmoninarum* infection than coho salmon with the transferrin genotype of 'CC.' It is possible that more fish which are less susceptible to the pathogen will survive, and produce offspring that are also less likely to become infected with *R. salmoninarum*, thereby reducing the prevalence of the disease over time.

An additional factor that may explain the minimal presence of *R. salmoninarum* in state fish hatcheries is the improved screening process for signs of diseases that was initiated at MDNR egg-collection weirs in the 1990s but expanded in the early 2000s. Once gametes were removed from adult *Oncorhynchus* spp., the fish were examined externally (presence of ulcers, furuncles, lesions, etc.) and internally (pale or swollen organs, granulomas, hemorrhages, etc.) by trained MDNR staff and experienced fish health professionals for signs of disease. If fish were suspected to be harboring *R. salmoninarum*, the gametes were not used for production. By using this method, fish affected by the acute form of BKD were removed from the brood-stock population, in addition to those that developed the chronic form of BKD (characterized by the formation of renal granulomas); thereby reducing the potential amount of *R. salmoninarum* that would be passed on to the progeny. Elliott et al. (1995) found significant differences in clinical BKD signs in Chinook salmon progeny from parent broodstock with a low prevalence of *R. salmoninarum* infection (low-BKD), which would mimic a chronic infection, when compared to a broodstock with a high prevalence of infection (high-BKD), which would be similar to an acute infection. Compared to the fish in the low-BKD group, a higher proportion of fish in high-BKD group had evidence of organ and tissue pathology, such as exophthalmia, corneal opacity, pale and/or frayed gills, fin erosion, swollen or mottled kidneys, enlarged spleens, and abnormal livers. While it is possible that the fish with low intensities of infection will not be detected by a screening method such as visual observation of the disease when compared to highly infected fish, fish with the low intensity of infection pose less of a risk of passing *R. salmoninarum* to their offspring. Similar to Elliott et al. (1995), Pascho et al. (1991) found that within the progeny that had positive ELISA results, most of the low- BKD group had low

intensity infections, while the majority of the fish in the high-BKD group had high intensity infections.

In this context, a long-term study (1993-2005) of *R. salmoninarum* infections in Chinook salmon in Idaho hatcheries has demonstrated the effectiveness of establishing a program such as screening the broodstock by an ELISA method and then culling based on the results, and continuing it on a yearly basis (Munson et al., 2010). It was found that the ELISA-based (as outlined in Munson et al., 2010) broodstock screening program reduced the prevalence, the intensity of infection, and mortality rates due to *R. salmoninarum* in Chinook salmon juveniles and broodstock. Our findings in Michigan corroborate with those of Munson et al. (2010) in Idaho.

Combining broodstock culling with the more stringent biosecurity measures implemented at the MDNR hatcheries as of 2002, have minimized, not only the vertical transmission from broodstocks, but also limited potential for horizontal transmission of *R. salmoninarum*. As recommended by Danner and Merrill (2006), each of the six state fish hatcheries utilizes separate nets, brushes, and buckets for each of the raceways that are cleaned and disinfected on a regular basis. Additionally, disinfecting footbaths and mats are placed at the entrance to all facilities to reduce the possible cross-contamination between facilities.

Another method of disease prevention that the MDNR implemented in 2002 is the use of the antibiotic erythromycin as a therapeutic treatment for hatchery salmon and trout. As it is extra-label use to use erythromycin as a therapeutic, the MDNR used it under the supervision of a veterinarian. *Renibacterium* salmoninarum is known to be susceptible to exposure to

erythromycin (Stoffregen et al., 1996). While MDNR uses erythromycin baths, Evelyn et al. (1986a) and Lee and Evelyn (1994) demonstrated that the intramuscular injection of erythromycin into broodstock fish can minimize the vertical transmission of *R. salmoninarum* into the ova. Additionally, Evelyn et al. (1986a) showed the antibiotic persisted within the eggs after attempts were made to leach it out; suggesting that it reduces the initial vertical transmission from parent to offspring; and potentially lowers the risk of horizontal transmission. It is likely that the erythromycin therapeutic baths that MDNR implemented on all egg lots acted in a similar fashion and further contributed to minimizing the potentials of vertical transmission. Unfortunately, as the MDNR could not risk the loss of valuable propagated fish, it was not possible to include a negative control (i.e., a hatchery with no newly implemented enhanced biosecurity measures) for the sake of comparison for this study.

Our finding of differences in *Renibacterium* salmoninarum prevalence among the three *Oncorhynchus* species matches the results of Starliper et al. (1997) and Beacham and Evelyn (1992). Starliper et al. (1997) demonstrated that strains of *R. salmoninarum* from coho salmon have the potential to be more harmful than strains of the bacteria from Chinook salmon. In six out of eight different salmonid hosts, a strain of *R. salmoninarum* from coho salmon from Manistee, Michigan was found to be more virulent than a strain from Chinook salmon from Manistee, Michigan (Starliper et al., 1997). Also, Starliper et al. (1997) found that coho and Chinook salmon were more susceptible to *R. salmoninarum* than rainbow (steelhead) trout. Furthermore, in a study by Beacham and Evelyn (1992), juvenile coho and Chinook salmon were infected with *R. salmoninarum* to better understand how the bacterium affected mortality rates, the mean time to death, and growth rates of the three species. It was concluded that

although they had a longer time to death, coho salmon had a higher percent of mortality than Chinook salmon (Beacham and Evelyn, 1992). A possible contributory factor that may help explain the greater prevalence of *R. salmoninarum* in coho salmon in this study is that broodstock of this species were only collected from streams in the Lake Michigan watershed, which overall had a greater prevalence of *R. salmoninarum* compared to fish collected from Lake Huron tributary streams.

The generally greater prevalence rate of *R. salmoninarum* in HB coho salmon versus MI coho salmon suggests that the strains may differ in their susceptibility to BKD. The studies of Withler and Evelyn (1990) documented that such variations in disease susceptibility can exist between strains of coho salmon. These investigators exposed two strains of coho salmon from British Columbia to *R. salmoninarum* to determine the likelihood of resistance to *R. salmoninarum*, as determined by survivability and time to death. The Kitimat River strain of coho salmon had greater survival and a longer time to death when compared to the Robertson Creek strain of coho salmon.

In addition to our study, several other studies have documented low occurrences of *R. salmoninarum* in *O. mykiss*, at least when compared to occurrence in brook trout, brown trout, Chinook salmon, and coho salmon (Bullock et al., 1971; Mitchum and Sherman, 1981; Mitchum et al., 1979; Hsu et al., 1991; Sakai et al., 1991; Jansson et al., 1996; Starliper et al., 1997). Additionally, Mitchum and Sherman (1981) found that *O. mykiss* had the lowest mortalities and the least severe clinical signs of BKD in a study investigating the horizontal transmission of *R. salmoninarum* from infected wild brook trout to newly stocked hatchery-raised brook trout, brown trout, and rainbow trout. Hsu et al. (1991) also found that steelhead in Lake Ontario had

lower prevalences of *R. salmoninarum* and also lower detectable antigen levels than coho or Chinook salmon among fish returning to the Salmon River Fish Hatchery (Altmar, New York) based on monoclonal-antibody-based ELISA. The prevalence of *R. salmoninarum* shedding in this study was low, with the exception of the HB coho salmon, which may be attributable to the finding that HB coho salmon had the highest overall prevalence of *R. salmoninarum* in the kidney and spleen of the parental broodstock.

The prevalence of shedding *R. salmoninarum* in coho salmon broodstocks in this study corresponded to the prevalence of the pathogen in the progeny. For example, in 2004, 72.5% of HB coho salmon were positive for *R. salmoninarum* and 52.5% of them were capable of shedding the bacteria. As a result, 43.3% of their progeny were positive for the pathogen. Interestingly, in 2005, the prevalence of *R. salmoninarum* in broodstock decreased to 18.3% with 11.7% of them shedding the bacteria. Consequently, none of the progeny were positive for *R. salmoninarum*. While vertical transmission clearly plays a role in the infection of the progeny, there are several other factors that can affect the prevalence of the disease as well, such as density of the fish in the raceway, environmental conditions (water temperature, dissolved oxygen levels, etc.), and biosecurity measures at the facility.

It is clear that female salmonids contribute to vertical transmission by having *R. salmoninarum*-infected ovarian fluid, but the role of male salmonids in vertical transmission is less understood. The shedding in this study occurred fairly equally between male and female fish; however, in the case of SRW-CHS and LMRW-STT, the males had a somewhat higher shedding prevalence than the females. Evelyn et al. (1986b) concluded that male coho salmon and steelhead did not play a significant role in vertical transmission as a result of their studies

examining infection rates in eggs fertilized with infected or non-infected milt. Based on our findings, it is at least plausible that male SRW-CHS and LMRW-STT could be contributing to the vertical transmission of *R. salmoninarum*, although the extent this is occurring is not known.

The overall decline of *R. salmoninarum* from 2001 to 2010 in the three feral broodstock and propagated fish stocks has shown that in addition to a possible heightened genetic resistance, preventative measures such as an improved screening process, broodstock culling, and enhanced biosecurity measures can be successful in reducing the prevalence of a pathogen in hatcheries and perhaps in returning broodstock from the Great Lakes. The broad decline in the prevalence of *R. salmoninarum* in the various fish species and stocks in this study is most likely due to a combination of improved visual inspections and culling conducted at the weirs, implementation of increased biosecurity measures at the hatcheries, reduced rearing stress, and iodophor and erythromycin disinfection of eggs. While the decline of *R. salmoninarum* in these three salmonid species is promising, BKD continues to be a potential problem in the LGL basin. Lake whitefish (Coregonus clupeaformis), which are in a closely related subfamily to salmonids (i.e., Coregoninae), have been shown to heavily infected with *R. salmoninarum*. Recently, Faisal et al. (2010) documented the presence of *R. salmoninarum* in approximately 66% of the lake whitefish populations sampled in northern Lake Huron by the Q-ELISA method described above, with predominant clinical signs of infection. This high infection prevalence in another susceptible fish species attests for the continuous strong presence of *R. salmoninarum* in the LGL ecosystem. It further strengthens the finding of this study that the decline of the bacterial presence in *Oncorhynchus* species is the result of disease management measures undertaken in Great Lakes salmonid gamete collection weirs and state fish hatcheries.

APPENDIX

Table 2.1. Enhanced biosecurity measures to control Bacterial Kidney Disease that have been implemented at Michigan Department of Natural Resources gamete-collecting weirs and hatchery facilities.

Biosecurity measure	Description
Clinical inspection	Examination for disease signs such as hemorrhages, exophthalmia, congested internal organs, and granulomas
Culling	Euthanasia of any individuals exhibiting the above signs
Egg disinfection	Disinfecting the external surface of the eggs to reduce the amount of bacteria
Hardening eggs in erythromycin-laden water	Water hardening the eggs (a necessary step for fertilization) in the antibiotic erythromycin, which *Renibacterium salmoninarum* is susceptible to
Regular screening	Frequent testing of propagated fish to determine if infection prevalence or mortality exceeded 0.05%
Antibiotic treatment	If prevalence indeed exceeded 0.05%, treatment with antibiotics under the investigational new animal drug exemption (INAD), as chosen by the antibiotic disc diffusion test

Table 2.2. The number of spawning *Oncorhynchus* spp. analyzed for the presence of *R. salmoninarum* antigens from 2001 to 2010. Hinchenbrooke coho salmon were not collected after 2007. ND = no data collected.

	Chinook salmon						Coho salmon						Steelhead		
	Little Manistee River Weir (LMRW-CHS)			Swan River Weir (SRW-CHS)			Michigan-adapted (MI-COS)			Hinchenbrooke (HB-COS)			Little Manistee River Weir (LMRW-STT)		
Year	# of ♀	# of ♂	TOTAL	# of ♀	# of ♂	TOTAL	# of ♀	# of ♂	TOTAL	# of ♀	# of ♂	TOTAL	# of ♀	# of ♂	TOTAL
2001	30	30	60	ND	ND	ND	19	19	38	25	24	49	ND	ND	ND
2002	30	30	60	30	29	59	83	82	165	28	28	56	ND	ND	ND
2003	30	30	60	17	17	34	30	30	60	24	24	48	ND	ND	ND
2004	60	60	120	560	0	560	58	59	117	60	60	120	30	30	60
2005	30	30	60	30	30	60	31	30	61	31	30	61	31	31	62
2006	30	30	60	30	30	60	30	30	60	30	30	60	30	30	60
2007	30	30	60	30	30	60	30	30	60	30	30	60	30	30	60
2008	30	30	60	30	30	60	30	30	60	ND	ND	ND	30	30	60
2009	50	50	100	50	50	100	50	50	100	ND	ND	ND	30	30	60
2010	50	50	100	50	50	100	50	50	100	ND	ND	ND	30	30	60
TOTAL	372	371	740	827	266	1093	411	410	821	228	226	454	211	211	422

Table 2.3. The number of propagated *Oncorhynchus* spp. reared in state fish hatcheries and tested for *Renibacterium salmoninarum* antigens prior to stocking. State fish hatchery facilities included the Platte River State Fish Hatchery (PRSFH), the Thompson State Fish Hatchery (TSFH), and the Wolf Lake State Fish Hatchery (WLSFH). *Species/strains were not reared at these locations at that year.

Year	Chinook salmon						Coho salmon		Steelhead	
	Little Manistee River Weir			Swan River Weir			Platte River Weir		Little Manistee River Weir	
	PRSFH	TSFH	WLSFH	PRSFH	TSFH	WLSFH	HB-PRSFH	MI-PRSFH	TSFH	WLSFH
2002	65	*	60	*	*	*	*	*	*	*
2003	60	*	60	60	60	*	60	60	*	*
2004	60	*	60	60	60	60	60	60	*	*
2005	60	*	60	60	60	60	120	120	60	60
2006	60	*	60	60	39	60	60	60	30	92
2007	60	*	60	60	60	60	59	60	119	159
2008	60	58	11	*	*	*	*	60	141	137
2009	60	*	60	60	60	*	*	60	126	120
2010	60	*	60	*	59	*	*	60	120	139
TOTAL	545	58	491	360	398	240	359	540	596	707

Table 2.4. Listing and description of models fit to the *R. salmoninarum* prevalence and shedding data. Analysis column indicates models fit only to propagated pre-stocking fingerling prevalence or broodstock gamete shedding data.

Model	Description	Analysis
Intercept	Common intercept; no change over time	All
Intercept + time	Common intercept; common change over time	All
Intercept + species × time	Common intercept; species specific change over time	All
Intercept + SS × time	Common intercept; species/stock/strain specific change over time	All
Intercept + SSH × time	Common intercept; species/stock/strain/ hatchery specific change over time	Propagated pre-stocking fingerlings
Intercept + sex × time	Common intercept; sex specific change over time	Broodstock shedding
Intercept + SX	Common intercept; species/sex specific change over time	Broodstock shedding
Intercept + SSX × time	Common intercept; species/stock/strain/sex specific change over time	Broodstock shedding
Species	Species specific intercept; no change over time	All
Species + time	Species specific intercept; common change over time	All
Species + species × time	Species specific intercept; species specific change over time	All
Species + SS × time	Species specific intercept; species/stock/strain specific change over time	All
Species + SSH × time	Species specific intercept; species/stock/strain/ hatchery specific changes over time	Propagated pre-stocking fingerlings
Species + sex × time	Species specific intercept; sex specific change over time	Broodstock shedding
Species + SX × time	Species specific intercept; species/sex specific change over time	Broodstock shedding
Species + SSX × time	Species specific intercept; species/stock/strain/ sex specific change over time	Broodstock shedding
SS	Species/stock/strain specific intercept; no change over time	All
SS + time	Species/stock/strain specific intercept; common change over time	All

Table 2.4 (cont'd)

Model	Description	Analysis
SS + species × time	Species/stock/strain specific intercept; species specific change over time	All
SS + SS × time	Species/stock/strain specific intercept; Species/stock/strain specific change over time	All
SS + SSH × time	Species/stock/strain specific intercept; species/ stock/strain/hatchery specific change over time	Propagated pre-stocking fingerlings
SS + sex × time	Species/stock/strain specific intercept; sex specific change over time	Broodstock shedding
SS + SX × time	Species/stock/strain intercept; species/sex specific change over time	Broodstock shedding
SS + SSX × time	Species/stock/strain specific intercept; species/stock/strain/sex specific change over time	Broodstock shedding
SSH	Species/stock/strain/hatchery specific intercept; no change over time	Propagated pre-stocking fingerlings
SSH + time	Species/stock/strain/hatchery specific intercept; common change over time	Propagated pre-stocking fingerlings
SSH + species × time	Species/stock/strain/hatchery specific intercept; species specific change over time	Propagated pre-stocking fingerlings
SSH + SS × time	Species/stock/strain/hatchery specific intercept; species/stock/strain specific change over time	Propagated pre-stocking fingerlings
SSH + SSH × time	Species/stock/strain/hatchery specific intercept; species/stock/strain/hatchery specific change over time	Propagated pre-stocking fingerlings
Sex	Sex specific intercept; no change over time	Broodstock shedding
Sex + time	Sex specific intercept; common change over time	Broodstock shedding
Sex + species × time	Sex specific intercept; species specific change over time	Broodstock shedding
Sex + SS × time	Sex specific intercept; species/stock/strain specific change over time	Broodstock shedding
Sex + sex × time	Sex specific intercept; sex specific change over time	Broodstock shedding

Table 2.4 (cont'd)

Model	Description	Analysis
Sex + SX × time	Sex specific intercept; species/sex specific change over time	Broodstock shedding
Sex + SSX × time	Sex specific intercept; species/stock/strain/sex specific change over time	Broodstock shedding
SX	Species/sex specific intercept; no change over time	Broodstock shedding
SX + time	Species/sex specific intercept; common change over time	Broodstock shedding
SX + species × time	Species/sex specific intercept; species specific change over time	Broodstock shedding
SX + SS × time	Species/sex specific intercept; species/stock/strain specific change over time	Broodstock shedding
SX + sex × time	Species/sex specific intercept; sex specific change over time	Broodstock shedding
SX + SX × time	Species/sex specific intercept; species/specific change over time	Broodstock shedding
SX + SSX × time	Species/sex specific intercept; species/stock/strain/sex specific change over time	Broodstock shedding
SSX	Species/stock/strain/sex specific intercept; no change over time	Broodstock shedding
SSX + time	Species/stock/strain/sex specific intercept; common change over time	Broodstock shedding
SSX + species × time	Species/stock/strain/sex specific intercept; species specific change over time	Broodstock shedding
SSX + SS × time	Species/stock/strain/sex specific intercept; species/stock/strain specific change over time	Broodstock shedding
SSX + sex × time	Species/stock/strain/sex specific intercept; sex specific change over time	Broodstock shedding
SSX + SX × time	Species/stock/strain/sex specific intercept; species/sex specific change over time	Broodstock shedding
SSX + SSX × time	Species/stock/strain/sex specific intercept; species/stock/strain/sex specific change over time	Broodstock shedding

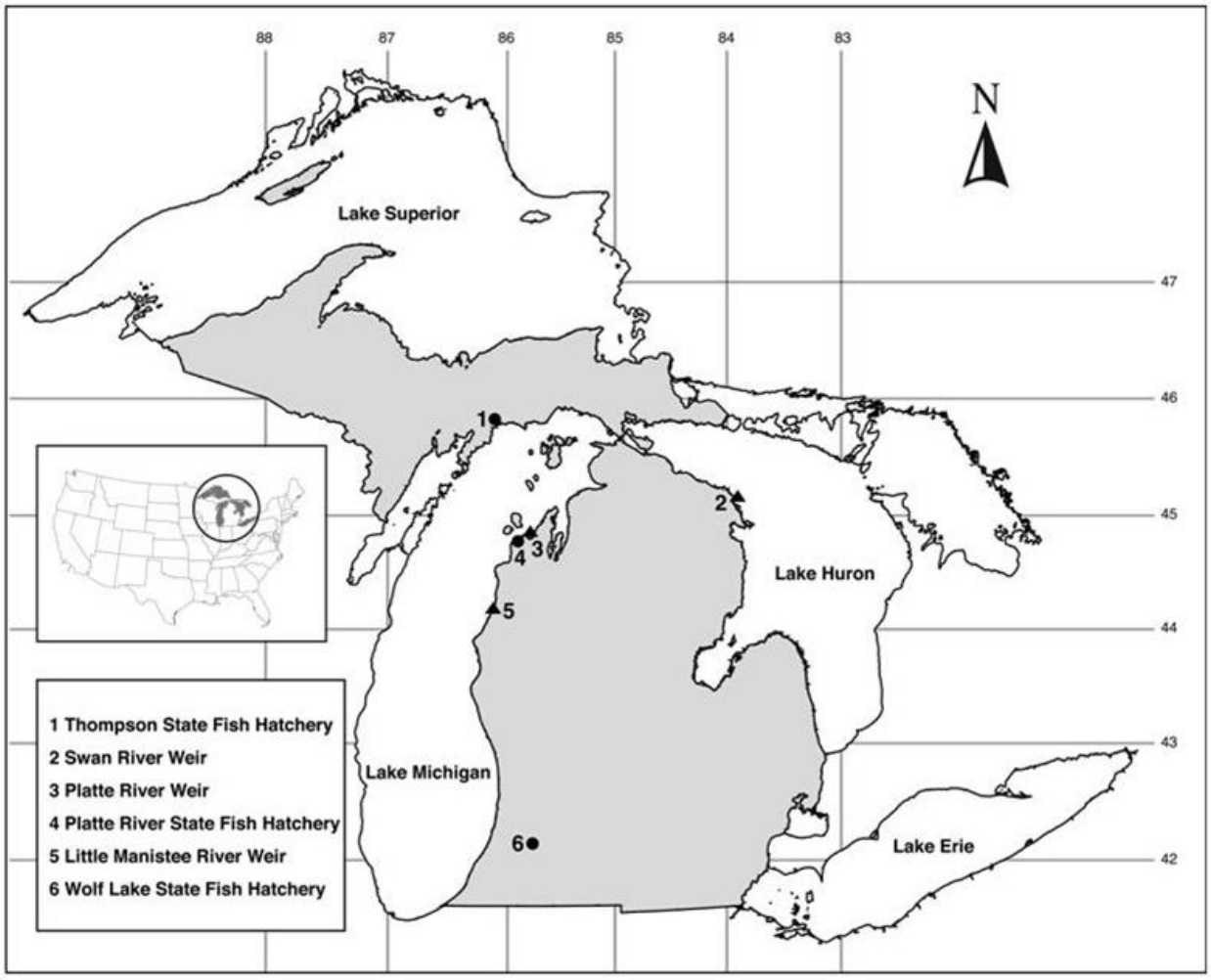

Figure 2.1. The Michigan Department of Natural Resources state fish hatcheries and gamete-collecting weirs where Chinook (*Oncorhynchus tshawytscha*) and coho salmon (*O. kisutch*) and steelhead (*O. mykiss*) were collected from 2001 to 2010: Little Manistee River Weir (44°11'51.66"N, 86°11'38.99"W), Platte River Weir and State Fish Hatchery (44°39'48.88"N, 85°56'13.20"W), Swan River Weir (45°24'10.09"N, 83°44'5.52"W), Thompson State Fish Hatchery (45°57'16.07"N, 86°15'29.36"W), and Wolf Lake State Fish Hatchery (42°17'40.14"N, 85°47'2.29"W).

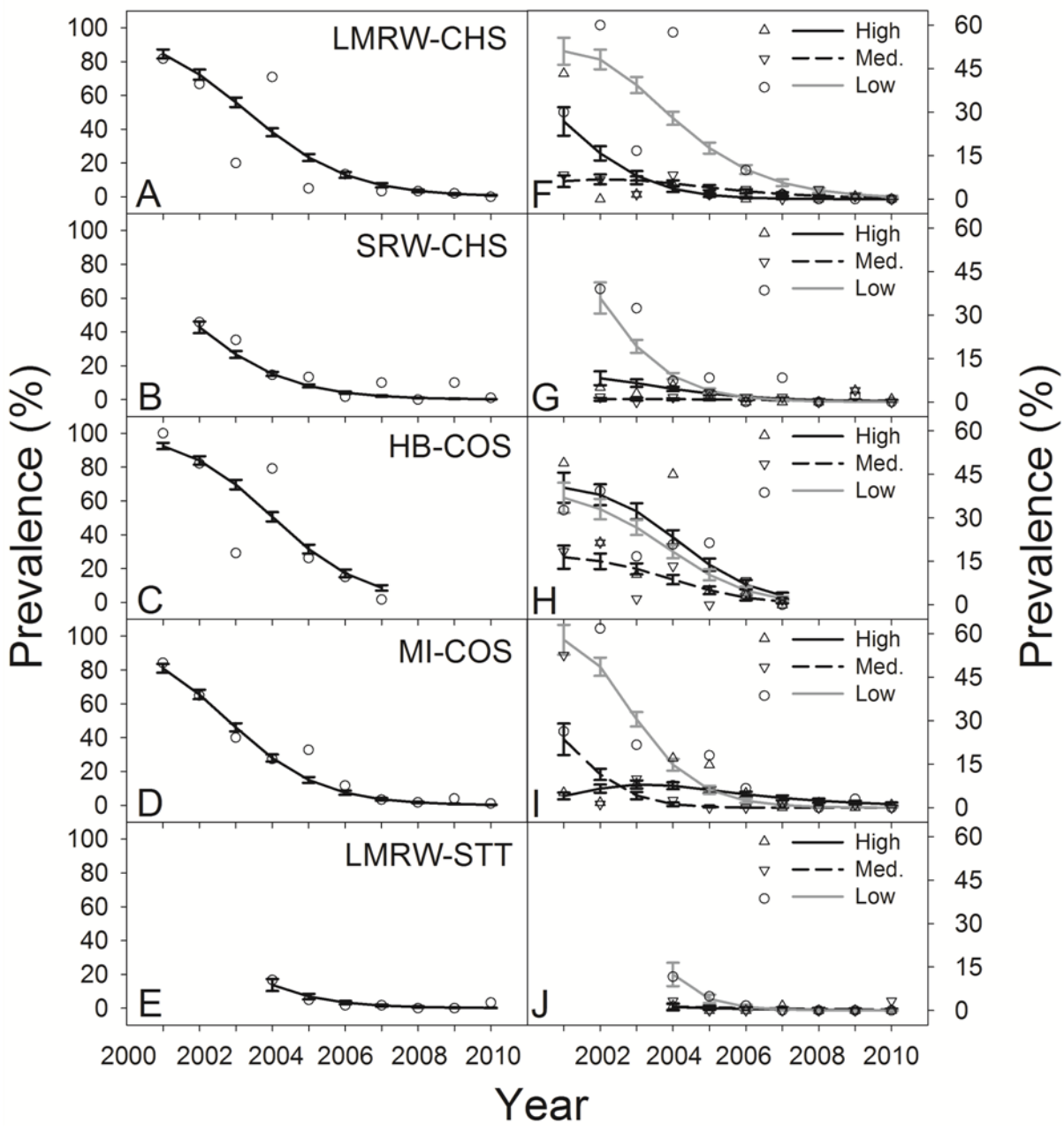

Figure 2.2. The prevalence of *R. salmoninarum* in Chinook salmon (*Oncorhynchus tshawytscha*) broodstock from the Little Manistee River Weir (LMRW-CHS) and the Swan River Weir (SRW-CHS), Hinchenbrooke coho salmon (*O. kisutch*) broodstock (HB-COS) and Michigan-adapted coho salmon broodstock (MI-COS) from the Platte River Weir, and steelhead (*O. mykiss*) broodstock from the Little Manistee River Weir (LMRW-STT) from 2001 to 2010. F-J: The low, medium, and high intensity levels of infection of *R. salmoninarum* in LMRW-CHS, SRW-CHS, HB-COS, MI-COS, and LMRW-STT from 2001 to 2010. The lines represent the logistic regression predicted prevalence and intensity levels of infection, while open circles and triangles denote the observed prevalence and intensity levels of infection.

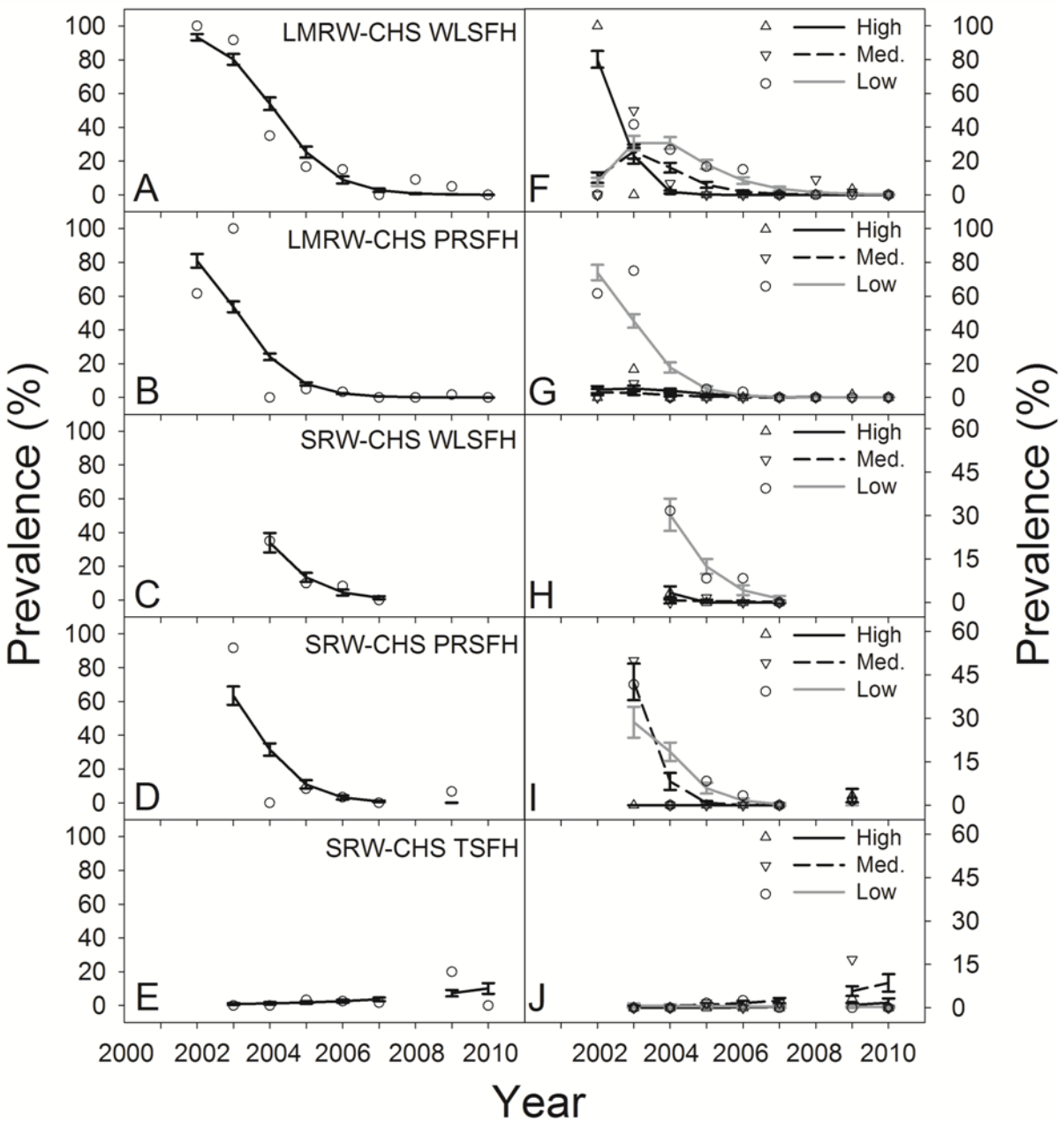

Figure 2.3. The prevalence of *R. salmoninarum* in Chinook salmon (*Oncorhynchus tshawytscha*) pre-stocking fingerlings propagated at the Wolf Lake State Fish Hatchery (WLSFH), the Platte River State Fish Hatchery (PRSFH), and the Thompson State Fish Hatchery (TSFH) from 2002 to 2010. Fingerlings are the progeny of broodstock spawned at the Little Manistee River Weir (LMRW) and the Swan River Weir (SRW). F-J: The low, medium, and high intensity levels of infection of *R. salmoninarum* in LMRW-CHS from WLSFH and PRSFH and SRW-CHS from the WLSFH, PRSFH, and TSFH from 2002 to 2010. The lines represent the logistic regression predicted prevalence and intensity levels of infection, while open circles and triangles denote the observed prevalence and intensity levels of infection.

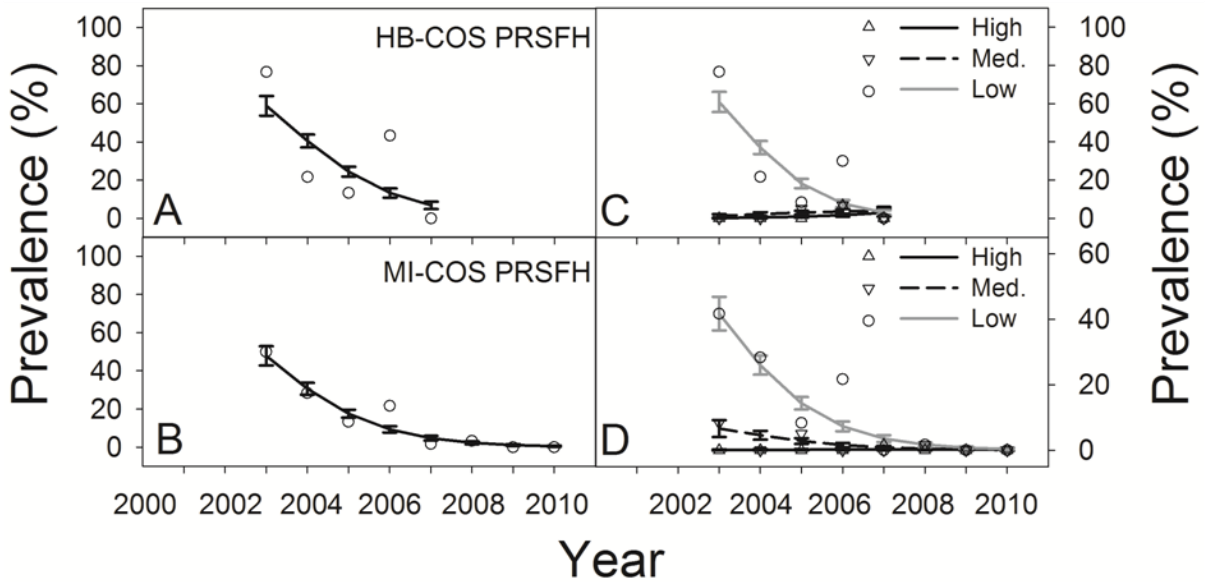

Figure 2.4. The prevalence of *R. salmoninarum* in the Hinchenbrooke strain (HB-COS) of coho salmon (*Oncorhynchus kisutch*) pre-stocking fingerlings and the Michigan-adapted strain of coho salmon (MI-COS) propagated at the Platte River State Fish Hatchery (PRSFH) from 2003 to 2010. C-D: The low, medium, and high intensity levels of infection of *R. salmoninarum* in HB-COS and MI-COS propagated at the PRSFH from 2003 to 2010. The lines represent the logistic regression predicted prevalence and intensity levels of infection, while open circles and triangles denote the observed prevalence and intensity levels of infection.

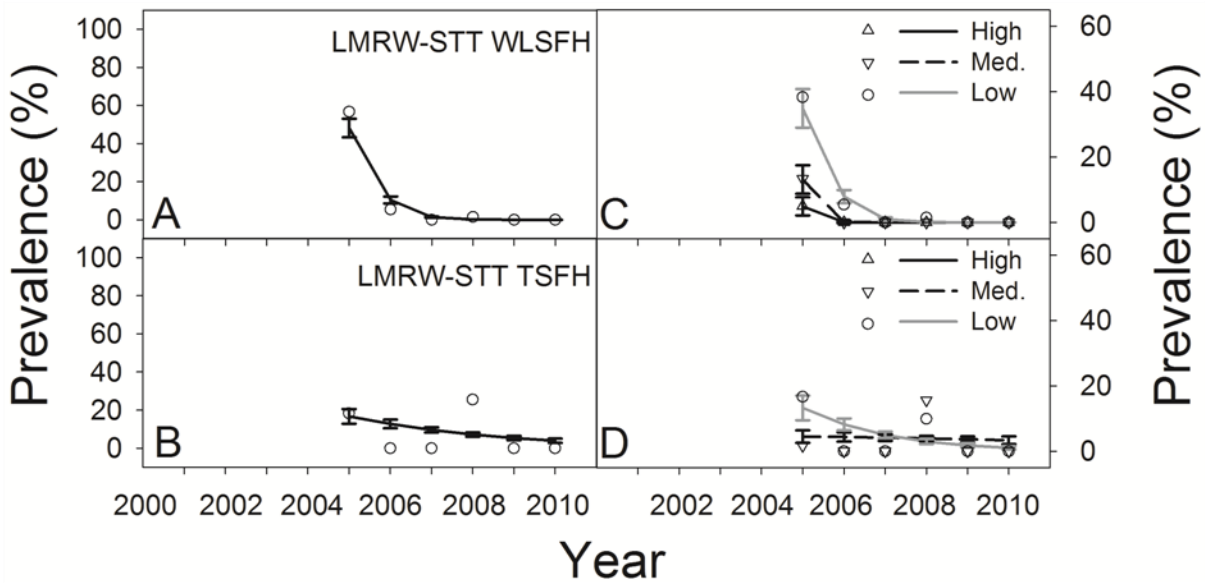

Figure 2.5. The prevalence of *R. salmoninarum* in steelhead (*Oncorhynchus mykiss*) pre-stocking fingerlings (LMRW-STT) propagated at the Wolf Lake State Fish Hatchery (WLSFH) and the Thompson State Fish Hatchery (TSFH) from 2005 to 2010. C-D: The low, medium, and high intensity levels of infection of *R. salmoninarum* in LMRW-STT propagated at the WLSFH and TSFH from 2005 to 2010. The lines represent the logistic regression predicted prevalence and intensity levels of infection, while open circles and triangles denote the observed prevalence and intensity levels of infection.

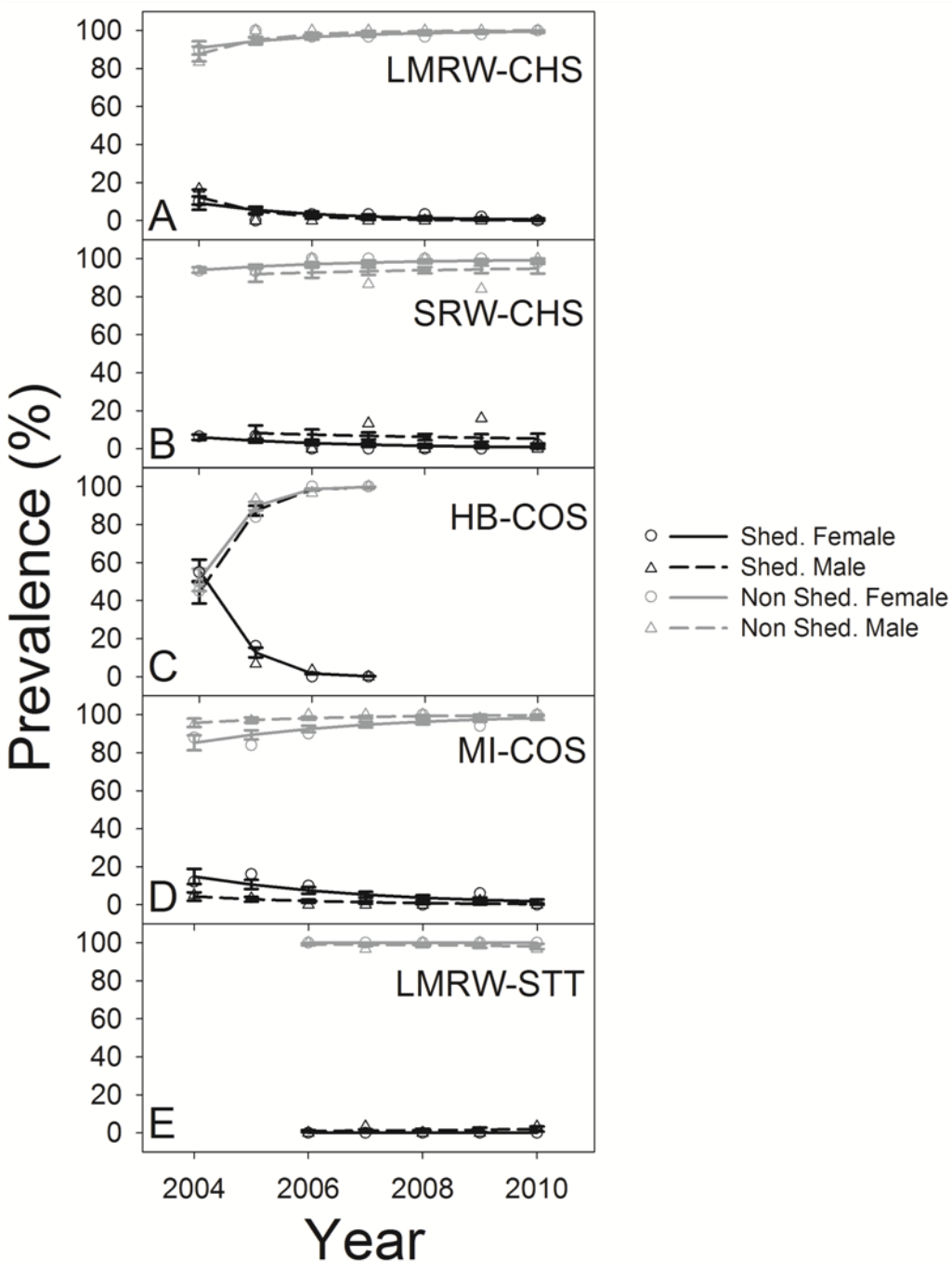

Figure 2.6. The prevalence of male and female fish that may be shedding *R. salmoninarum* and are not shedding *R. salmoninarum* for (A) Chinook salmon (*Oncorhynchus tshawytscha*) broodstock from the Little Manistee River Weir; (B) Chinook salmon broodstock from the Swan River Weir; (C) Hinchenbrooke coho salmon (*O. kisutch*) broodstock from the Platte River Weir; (D) Michigan-adapted coho salmon broodstock from the Platte River Weir; (F) and steelhead (*O. mykiss*) broodstock from the Little Manistee River Weir. The lines represent the logistic regression predicted prevalence and intensity levels of infection, while open circles and triangles denote the observed prevalence and intensity levels of infection.

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Chapter 3

The Use of Non-lethal Samples for the Detection of *Renibacterium salmoninarum* in Chinook salmon, *Oncorhynchus tshawytscha* (Walbaum)

1. Abstract

Bacterial kidney disease, caused by *Renibacterium salmoninarum*, threatens salmonid populations in the Northern hemisphere. Most fishery regulatory authorities require continuous monitoring of the disease in hatcheries and spawning runs. As per the diagnostic protocols of the World Organisation for Animal Health and the American Fisheries Society-Fish Health Section, lethal sampling of visceral organs is used from a set number of fish, which depends on the assumed disease prevalence. Non-lethal sampling would be a preferable alternative, especially in the case of valuable broodstock and endangered species. In this study, non-lethal sampling methods were evaluated for their ability to detect *R. salmoninarum* in experimentally infected Chinook salmon (*Oncorhynchus tshawytscha*). Non-lethal (e.g., blood, mucus, and a urine/feces mixture) and lethal (e.g., a kidney and spleen homogenate) samples were collected from 178 Chinook salmon that were experimentally infected with *R. salmoninarum*. *Renibacterium salmoninarum* was detected in all samples by culture on modified kidney disease medium, nested polymerase chain reaction (nPCR), and quantitative enzyme-linked immunosorbent assay (ELISA), although detection depended on sampling period. The sensitivity, specificity, and accuracy of the lethal and non-lethal samples in detecting the presence of *R. salmoninarum* were calculated using receiver operator characteristic (ROC) analyses using the assumption that all infected fish were positive for *R. salmoninarum*. Non-lethal samples did detect *R. salmoninarum*; however, the level of sensitivity and accuracy depended upon the exposure route and the subsequent disease course. ROC analyses revealed that the uro-fecal sample has the greatest potential for non-

lethal sampling compared to mucus and blood samples. Also, combining the nPCR and ELISA data from the lethal samples with the uro-fecal samples has the potential to be the best strategy for detecting *R. salmoninarum* prevalence and intensity in a population.

2. Introduction

Bacterial pathogens frequently threaten the health of both wild and aquacultured fishes worldwide (Meyer, 1991; reviewed in Austin and Austin, 2007). To prevent the spread of bacterial infections in aquaculture facilities or for stocking purposes in public waters, health inspections are required to effectively manage disease outbreaks within infected fish populations (World Organisation for Animal Health (WOAH), 2013). The current methods for detecting many bacterial fish pathogens, as outlined by the American Fisheries Society-Fish Health Section (AFS-FHS) Blue Book (2012) and the Manual of Diagnostic Tests for Aquatic Animals (WOAH, 2012), require the use of lethal sampling techniques, and therefore, the sacrifice of individual fish. In order to provide a 95% confidence of detecting a pathogen at an assumed minimum incidence of 5%, at least 60 fish must be sampled lethally from the overall population, or with a minimum incidence of 10%, at least 30 fish (Ossiander and Wedemeyer, 1973; Hnath, 1993; AFS-FHS Blue Book, 2012). In some instances, lethal sampling of more than 60 fish is required because it increases the likelihood of detecting the pathogen (Fenichel et al., 2008). Sacrificing this number of fish from a threatened or endangered species or from a broodstock population containing irreplaceable individuals with unique traits is often impractical (Powell et al., 2005). This matter is complicated by the fact that results of each of

the diagnostic assays commonly used in BKD diagnosis depend primarily on the disease progression and course (Faisal and Eissa, 2009; Schulz, Chapter 5).

Several studies on fish pathogens have generated encouraging results regarding the use of non-lethal sampling in disease diagnosis. For example, *Yersinia ruckeri*, the causative agent of Enteric Redmouth Disease, has been detected from fecal material and blood collected non-lethally (Rodgers, 1992; Altinok et al., 2001-blood), and *Aeromonas salmonicida*, the etiological agent of furunculosis was recovered non-lethally from the gills, mucus, and blood of infected fish (Benediktsdóttir and Helgason, 1990; Cipriano et al., 1996; Cipriano et al., 1997; Klinger et al., 2003). In addition, viral pathogens have also been detected in non-lethally collected tissues, such as Infectious Pancreatic Necrosis Virus from the pectoral fin (Bowers et al., 2008) and Infectious Salmon Anemia Virus from the blood of infected fish (Giray et al., 2005).

Renibacterium salmoninarum, the causative agent of Bacterial Kidney Disease (BKD), is unique in terms of its pathogenesis. BKD can run an acute course with mortalities occurring, or more often, it can run a subclinical disease course with a low infection prevalence and intensity. The strong host granulomatous reaction against *R. salmoninarum* sequesters the bacteria, and unless the tissues containing the bacteria are collected, the bacterium presence may be undetected (MacLean and Yoder, 1970; DeKinkelin, 1974; Kimura and Awakura, 1977; Hoffmann et al., 1984; Sanders and Barros, 1986; Holey et al., 1998). Although both horizontal and vertical routes have been identified for *R. salmoninarum* transmission, details pertaining to the spread of the bacterium within the fish body remains largely unknown, despite genuine research efforts. This fact constitutes an obstacle for the proper diagnosis of the bacterium, particularly in latently infected fish.

To this end, this study was designed to 1) determine if *R. salmoninarum* could be detected in samples that are collected non-lethally (blood, mucus, and uro-fecal samples), 2) evaluate how exposure route and the resultant disease course impacts the detection of *R. salmoninarum* in all sample types, 3) compare the efficacy of non-lethally collected samples to those collected lethally (i.e., spleen and kidney samples), 4) determine the best combination of sample type and diagnostic method to detect *R. salmoninarum*, and lastly 5) determine the number of fish that would be required for non-lethal testing, while still maintaining the efficacy of lethal testing, with an assumed disease prevalence of 5 and 10%.

3. Materials and methods

3.1. Source of fish

Five hundred Chinook salmon (~8 months old) were acquired from the Michigan Department of Natural Resources (MDNR) Wolf Lake State Fish Hatchery (Van Buren County) and transferred to the University Research Containment Facility at Michigan State University for experimental challenges. A sub-sample of the fish (n=60) were sacrificed and screened for the presence of *R. salmoninarum*. All fish were negative for *R. salmoninarum* by the quantitative enzyme-linked immunosorbent assay (Q-ELISA) as outlined in Pascho and Mulcahy (1987), with minor modifications (see description below). Fish were fed to satiation twice a day with BioTrout 2.0 mm pellets (Bio-Oregon, Westbrook, Maine) and were held in a 600 L continuous

flow-through tank with chilled freshwater at an average temperature of 12 ± 2.0°C, until needed for experimental infections.

3.2. Challenge by immersion bath

To mimic the natural route of infection, fish were exposed to *R. salmoninarum* via an immersion bath. Cryo-preserved *R. salmoninarum* (ATCC #33209) was revived on modified kidney disease medium (MKDM; Faisal et al., 2010), incubated at 15°C for 14 d, purity verified, and then a single colony was inoculated into a 7 ml aliquot of MKDM broth (x4) and incubated at 15°C for 7 d. Twenty µl from each of the four broth cultures were then sub-cultured onto trypticase soy agar (TSA) and MKDM to verify purity, and the remaining broth was added to 1900 ml of fresh MKDM broth and incubated at 15°C on a Thermolyne Nuova stir plate (Thermo Fischer Scientific, Inc., Waltham, MA) at approximately 50 rpm in a 2 L Celstir Spinner Flask (Wheaton, Millville, NJ). After 14 days of incubation, the broth culture was centrifuged in a Hermle Z382K centrifuge (Labnet International, Inc., Woodbridge, NJ) at 4300 rpm for 10 min, the supernatant was discarded, and the bacterial pellet was re-suspended in 0.85% saline solution, which was repeated a total of 3 times. The number of colony forming units per ml (CFU ml^{-1}) within the *R.salmoninarum*-0.85% saline suspension was then determined using 10-fold serial dilutions and plate counts via drop culture in triplicate. One third of the *R. salmoninarum* suspension were then added to 3.5 L of autoclaved tank water supplemented with 0.85% NaCl (total final volume of 4 L) for a final concentration of ~2.3×10^7 CFU ml^{-1}. Each of the three suspensions was placed into a separate, sterile 5 gallon bucket that was heavily

aerated, to which 42 Chinook salmon (12.6 g ± 4.4 g; 10.7 cm ± 1.5cm) were added. Each bucket was then covered and fish were immersion exposed for one hour. One bucket of 42 fish served as a negative control, with fish being immersed in the same fashion in a sterile saline suspension. Fish and the *R. salmoninarum* suspension were then poured into their empty respective 74 L tanks (12 ± 2.0°C) and water for flow-through system (1.09 L min^{-1}) was resumed. All tanks were monitored daily for mortalities.

3.3. Challenge by intraperitoneal injection

To generate an acute course of BKD, fish were intraperitoneally (i.p.) injected with *R. salmoninarum*. *Renibacterium salmoninarum* was revived and cultured as described above, with the exception that the bacterial suspension was grown in 1 L of MKDM broth. After 14 days of incubation, the broth culture was centrifuged and washed as described above, and the remaining pellet was re-suspended into 101 ml of sterile saline, for a final concentration of 2.1 x 10^{10} cfu ml^{-1}. Fish (61.1 g ± 4.8 g; 18.8 cm ± 0.6 cm) were anesthetized with 100 mg L^{-1} of sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Inc., Redmond, Washington) for approximately 10-15 sec, and were then i.p. injected with 200 µl of the bacterial suspension and revived by placing them in freshwater in their respective 225 L tanks, with a flow rate of 1.62 L min^{-1} and water temperature of 12 ± 2.0°C. This was done in accordance with the Michigan State University International Animal Care and Use Committee (Animal Use Form # 02/10-013-00). Three replicates of 25 fish were

infected in this fashion. One tank of 25 fish served as a negative control, whereby fish were injected with 200 µl of sterile saline. All tanks were monitored daily for mortalities.

3.4. Sampling procedures

Fish challenged with *R. salmoninarum* via immersion were sampled every 21 days (n=21), culminating in a total of 6 sampling periods for a total of 115 fish sampled from July to October 2010. Fish that were challenged via i.p. injection with *R. salmoninarum* were sampled every 7 days (n=15) over 5 sampling periods, for a total of 63 fish sampled during May and June 2011. Prior to non-lethal sample collection, fish were anesthetized with a dose of 100 mg L^{-1} of sodium bicarbonate-buffered MS-222 (Argent). Once anesthetized, mucus, blood, and a urine-feces mixture (which will be referred to as "non-lethal" samples) were taken from each fish. Mucus was collected by gently running an individual plastic cover slip in a posterior direction from the left pectoral fin to the caudal fin. Mucus was then placed into a sterile 1.5 ml microcentrifuge tube and frozen at -80°C until diagnostic assays were performed. Mucus was tested for the presence of *R. salmoninarum* by culture, nested polymerase chain reaction (nPCR), and Q-ELISA (see descriptions below).

Blood samples were collected by venipuncture of the caudal vein using a sterile needle and syringe, which was subsequently stored in a 1.5 ml microcentrifuge tube at -80°C until diagnostic assays were performed. Prior to cryopreservation, 5 µl of blood was inoculated onto MKDM agar plates (see description below). Blood was tested for the presence of *R. salmoninarum* by culture and nPCR.

A urine-feces mixture (referred to as uro-fecal) was obtained by gently pressing the ventral abdomen in an anterior to posterior direction and collecting the mixture in a sterile 1.5 ml microcentrifuge tube. Any urine-feces mixture that was expressed was collected in the microcentrifuge tube and stored at -80°C until assayed. The uro-fecal samples were tested for *R. salmoninarum* by culture, nPCR, and Q-ELISA.

After non-lethal sample collection was completed, fish were euthanized with a lethal dose of 250 mg L^{-1} of sodium bicarbonate-buffered MS-222 (Argent). A thorough internal and external examination for gross signs of disease was performed. Kidney and spleen samples (referred to as kidney/spleen or lethal samples) were collected with individual sterile forceps and scissors, placed in individual 1.5 ml microcentrifuge tubes, and stored at -80°C until diagnostic assays were performed. Lethal samples were tested for *R. salmoninarum* by culture, nPCR, and Q-ELISA.

3.5. Bacterial culture and isolation

Renibacterium salmoninarum isolation was performed by streaking 1 µl of the target tissue onto MKDM agar plates using sterile disposable inoculating loops and incubating the plates under aerobic conditions at 15°C for a total of 42 days. Five microliters of blood was inoculated directly onto MKDM plates at the time of necropsy. The mucus, uro-fecal, and kidney/spleen samples were diluted 1:10 (weight/volume) in sterile phosphate buffered saline (PBS; pH 7.2) and suspended by repeated expulsion through a sterile pipette. Each tissue suspension was then diluted via 10- fold serial dilutions and 10 µl of each dilution (e.g.,

undiluted to 10^{-8}) was dispensed onto the MKDM plates. Plates were incubated at 15°C and were observed for typical *R. salmoninarum* growth every 7 days for a total of 6 weeks. Colonies were observed under a dissecting microscope for typical *R. salmoninarum* morphological characteristics: convex, cream-colored, round, and smooth (Austin and Austin, 2007). Colonies that fit this criterion were assessed by additional biochemical testing, which included Gram stain, cytochrome oxidase, and catalase reaction, which are key biochemical tests used to identify *R. salmoninarum* (Sanders and Fryer, 1980). Colonies that were Gram positive, cytochrome oxidase negative, and catalase positive were then confirmed molecularly via nPCR (see below).

3.6. Extraction of DNA

The DNA from blood and mucus samples was extracted using a DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA) according to the manufacturer instructions for nucleated blood samples. The DNA from kidney/spleen samples were also extracted using the DNeasy® Blood and Tissue Kit (Qiagen), but the protocol for animal tissue was followed. DNA from the uro-fecal samples was extracted using the QIAamp® DNA Stool Mini Kit (Qiagen), according to the protocol of the manufacturers. In all cases, the pre-treatment for gram-positive bacteria (including the lysis buffer) was applied to all of the sample types. After extraction, the DNA was quantified with the Qubit® Fluorometer (Life Technologies, Grand Island, NY) and then diluted to a 20 ng μl^{-1} concentration.

3.7. Nested PCR

The nPCR method and primers recommended by Pascho et al. (1998) were used initially, with minor modifications. To optimize the protocol to the laboratory's conditions, the annealing temperature was changed to 60°C for DNA extracted from pure bacterial cultures and kidney/spleen samples (based upon optimization experiments). Additionally, the total reaction volume was reduced from 50 µl to 25 µl, consisting of 1 µl each of template DNA (20 ng total), forward primer (10 µmol), and reverse primer (10 µmol), as well as 12.5 µl of GoTaq® Green Master Mix (Promega Corp., Madison, WI) and 9.5 µl of nuclease-free water. The controls were composed of a PCR mixture containing water instead of DNA template (negative control) and DNA from a pure culture of *R. salmoninarum* ATCC #33209 strain (positive control). Five microliters of the nPCR products and controls were mixed with 1 µl of SYBR Green II RNA Gel Stain (Cambrex Bio Science Rockland, Inc., East Rutherford, NJ) and loaded into a gel consisting of 2% Ultra Pure™ agarose (Invitrogen, Grand Island, NY). Each electrophoresis gel included 5 µl of a 1 Kb plus ladder (Invitrogen) mixed with 1 µl of 6X gel loading dye (New England Bio Labs, Inc., Ipswich, MA). Gels were run at 100 v for 35 minutes in 1X Tris-Acetate Buffer (Sigma-Aldrich Corp., St. Louis, MO) in a Gel XL Ultra V-2 electrophoresis box (Labnet) and a Sub-Cell GT electrophoresis box (Bio Rad Laboratories, Inc., Hercules, CA). Gels were visualized with a Canon G10 camera and UV Trans-Illuminator. Samples were considered positive for *R. salmoninarum* when a 320-bp band was present (Pascho et al. 1998).

Prior to conducting nPCR on the blood, mucus, and uro-fecal samples, representative samples that were known to be *R. salmoninarum*-positive were first analyzed to assess the

suitability of the protocol described above. Subsequently, it was determined that DNA extracted from the different tissue types had different optimal annealing temperatures. While the nPCR for the kidney/spleen samples was performed as described above, the annealing temperature for the blood samples was modified to 58.5°C, the mucus samples were modified to 59°C, and the uro-fecal samples were modified to 56°C.

3.8. Q-ELISA

The general Q-ELISA protocol outlined in Pascho and Mulcahy (1987), with modifications recommended by Gudmundsdóttir et al. (1993) and Olea et al. (1993), was used to assess *R. salmoninarum* antigens in the mucus, uro-fecal, and kidney/spleen samples. Due to the small amount of tissue available from each fish, its need to be used in several diagnostic assays, and a minimum volume of diluted tissue required for each assay, different dilution factors were determined for the various sample types, as described below. Prior to the Q-ELISA procedure, kidney/spleen tissue samples were diluted 1:8 (w:v) with Hank's Balanced Salt Solution (HBSS; Sigma) and stomached on high speed for two minutes with the Biomaster Stomacher (Wolf Laboratories Limited, Pocklington, York, UK). An aliquot of 250 µl of stomached kidney/spleen samples were dispensed into 1.5 ml microcentrifuge tubes containing 250 µl of phosphate buffered saline with Tween-20 (PBS-T20; Sigma) and 5% goat serum (Sigma) and 50 µl of CitriSolv (Fisher Scientific, Pittsburgh, PA). The purpose of the CitriSolv solvent is to dissolve and remove lipids from the aqueous supernatant (Gudmundsdóttir et al., 1993), while the introduction of 5% goat serum increases the sensitivity of the assay (Olea et al., 1993). Samples

were vortexed for approximately 10 sec, heated at 100°C for 15 min, and then centrifuged at 14,000 rpm for 10 min. The aqueous supernatant of each sample was used for Q-ELISA testing.

Mucus samples were diluted 1:4 (w:v) with sterile HBSS and were vortexed thoroughly to ensure a homogeneous mixture. The mucus samples were then aliquoted (250 µl) into 1.5 ml microcentrifuge tubes containing 250 µl of PBS-T20 with 5% goat serum and 50 µl of CitriSolv and processed as described above. The uro-fecal samples were diluted 1:100 (w:v) with sterile HBSS, vortexed to homogenize the mixture, and then subsequently centrifuged at 14,000 rpm for 5 min to pellet any inhibiting factors. The supernatant from the uro-fecal samples were then aliquoted (250 µl) into 1.5 ml microcentrifuge tubes containing 250 µl of PBS-T20 with 5% goat serum and 50 µl of CitriSolv and processed as described above.

The positive-negative cutoff absorbance for the samples was 0.10. Samples that tested positive were assigned the following antigen levels: low (0.10-0.199), medium (0.20-0.999), and high (≥1.00), as recommended by Meyers et al. (1993) and Pascho et al. (1998). Each assay included two negative controls, a negative fish tissue sample and a dilution buffer; and two positive controls, a positive fish tissue sample and a *R. salmoninarum* positive control (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

3.9. Statistical analyses

Receiver operating characteristic (ROC) analyses were used to assess the analytical sensitivity, specificity, and accuracy of the non-lethal samples to predict the presence of *R. salmoninarum*. ROC analyses compared the results of the non-lethal samples to a 'gold

standard' and calculated the rates of false positives (FP), false negatives (FN), true positives (TP), and true negatives (TN), which were then used to determine sensitivity, specificity and accuracy (Zhu et al., 2010). All calculations were performed in SAS 9.3 (SAS Institute, Inc., Cary, NC). In both challenges, fish were exposed to or injected with virulent *R. salmoninarum*; therefore, the 'gold standard' was based on the assumption that all infected fish were truly positive. Sensitivity was considered the proportion of true positives that were correctly identified by the non-lethal samples and was calculated by dividing the number of true positive assessments by all of the positive assessments:

$$Sensitivity = \frac{TP}{(TP + FN)}$$

Sensitivity is often an indicator of how capable a test is at correctly detecting a pathogen; therefore, tests with high sensitivity capture most of the positive outcomes and are usually ideal to screen for a pathogen (Zhu et al., 2010).

The specificity was also calculated, which was the proportion of true negatives that were correctly identified by the non-lethal samples, and was calculated by dividing the number of true negative assessments by all of the negative assessments:

$$Specificity = \frac{TN}{(TN + FP)}$$

Specificity is an indicator of how well a diagnostic test identifies the normal, non-diseased conditions; therefore, tests with high specificity imply that a test is highly reliable for identifying true negatives.

Accuracy was the proportion of true results (positive and negative) that were correctly identified by the non-lethal samples and was calculated by dividing the number of correct assessment by all of the assessments:

$$Accuracy = \frac{(TN + TP)}{(TN + TP + FN + FP)}$$

Accuracy is a measure of the reliability of a diagnostic test, combining the sensitivity and specificity to demonstrate how well it identifies the true positives and negatives. However, a test with high sensitivity and specificity does not always have a high accuracy rate, especially when diagnosing rare diseases (Zhu et al., 2010).

In addition to sensitivity, specificity, and accuracy, the area under the curve (AUC) metric was also determined. Area under the curve values are typically calculated by plotting the true positive rate against the false positive rate for different cut-off points of the diagnostic test; however, in binary tests, where there is only one cut-off point (positive or negative), the AUC is calculated by adding the sensitivity and specificity together and dividing by two (Cantor and Kattan, 2000). In this study, the AUC measures the ability of the non-lethal samples to correctly identify individuals with and without disease. Area under the curve values that are 1.0 imply that the diagnostic test has perfect discrimination (i.e., always correctly identifies true positives and true negatives), while AUC values that are > 0.9 are considered "outstanding" discrimination, 0.9-0.8 are "excellent," 0.7-0.8 are "acceptable," and ≤ 0.6 are considered to be no better than chance alone (Hosmer and Lemeshow, 2000; Lalkhen and McCluskey, 2008; Zhu et al., 2010).

The accuracy, sensitivity, specificity and AUC values were calculated for the lethal and non-lethal samples from the immersion challenge, the injection challenge, and also for the two

challenges combined together. Additionally, for the immersion and injection challenge, the accuracy, sensitivity, specificity, and AUC values were determined for each particular non-lethal tissue overall, and during the various stages of disease progression. An individual sample was considered positive for *R. salmoninarum* if it was positive by at least one of the diagnostic tests (e.g., culture, ELISA, and/or nPCR).

Using the assumption that a naturally infected population would be comprised of fish in various stages of disease progression (chronic and sub-acute), the number of non-lethal samples that would be necessary to collect from a population of wild fish to detect BKD was estimated. The immersion and i.p. injection challenge data was used to create a simulated population that would be randomly composed of individuals from both challenges, but with specified numbers of both disease courses (e.g., 100% sub-acute, or 40% sub-acute and 60% chronic). To create each simulated population, stratified random sampling was used, using PROC SURVEYSELECT in SAS to randomly draw a specified percentage of individuals from each exposure regime, while maintaining a consistent representation across days of exposure. The simulated population was then used to calculate sensitivity, specificity and accuracy values.

The simulated populations were designed to represent the range of possible scenarios of sub-acute and chronic composition in a population. Analysis began with 100% sub-acute, and then incrementally decreased the sub-acute cases by 10% while simultaneously incrementally increasing the chronic cases by 10% until 100% chronic was reached. For each scenario, experimental data was resampled using the stratified random sampling technique 50 times and sensitivity, specificity and accuracy values were calculated each time. The average sensitivity, specificity, and accuracy for 50 iterations for each scenario was used, and FreeCalc

2.0 (AusVet Animal Health Services, Lyon, France) estimated the sample size for a population 10,000 fish, with type I and II error levels set at 0.05 and a minimum estimated prevalence (MEP) of 5 and 10%. An MEP of 5 and 10% was chosen based on data reflecting the current and historical prevalence of *R. salmoninarum* in fish populations in Michigan (Schulz, Chapter 2). FreeCalc 2.0 calculates the number of individuals that must be tested in order to provide evidence, at a specified level of confidence, that disease is not present (Cameron and Baldock, 1998; Cameron, 2001). Therefore, for this study, the number of non-lethal samples required for testing to obtain the same 95% level of confidence that lethal samples afford was estimated.

4. Results

4.1. Detection in non-lethally collected samples

Renibacterium salmoninarum was detected in both the lethal and non-lethal samples recovered from fish that were exposed to *R. salmoninarum* by the immersion and i.p. injection methods (Table 3.1). The uro-fecal samples had the highest prevalence of *R. salmoninarum* in both of the challenges (Table 3.1). In the injection challenge, there was a greater prevalence of *R. salmoninarum* in the blood samples compared to the mucus samples; conversely, the mucus samples had a slightly higher detection of *R. salmoninarum* than the blood samples in the immersion challenge (Table 3.1).

4.2. Effect of exposure route

In the injection challenge, the fish developed a sub-acute course of BKD, with some of the challenged fish surviving through the end of the observation period. As early as day 1 post-infection (p.i.), characteristic clinical signs of BKD were observed (Figure 3.1A-E). Disease signs included diffuse petechial hemorrhages covering the body of the fish (Figure 3.1A), severe gill pallor (Figure 3.1B), corneal opacity, frequently associated with ocular hemorrhage (Figure 3.1C), whitish, false membranes covering the liver (Figure 3.1D), and whitish, granulomatous-like lesions in the spleen and kidney (Figure 3.1E).

Differences were noted throughout the study period, with fewer clinical signs being observed during early sampling periods. For example, on days 1 and 8 p.i., ocular hemorrhage and few external petechial hemorrhages were noted, along with mild heart and liver pallor, but only in 13-27% of the fish. However, on day 15 p.i., the formation of ascites was first observed, as well as heart and liver pallor, white granulomatous-like lesions in the kidney, and whitish, false membranes covering the spleen in 73% of the fish. On days 22 and 29 p.i., gill pallor, petechial hemorrhages throughout the external body, exophthalmia, ascites, liver and heart pallor, white granulomatous-like lesions in the spleen and kidney, and false membranes covering the liver were observed in 100% of the fish. Outside of the fish euthanized for regular sampling, mortalities started occurring as early as day 9 p.i. with a total of 7 mortalities occurring throughout the 29-day study period. Mortalities also exhibited the external and internal clinical signs described above. There were no clinical signs observed in the control (i.e., non-infected) fish.

At each sampling period in the injection challenge, *R. salmoninarum* was detected in all of the individual lethal samples, for a prevalence of 100%. However, depending on the non-lethal tissue, there was more variation in the presence and detection of *R. salmoninarum* at the sampling periods (Table 3.1). The uro-fecal samples were the most consistent non-lethal sample for *R. salmoninarum* detection, although there was a decrease in the prevalence on day 29 p.i. (Table 3.1). The blood samples regularly detected *R. salmoninarum* throughout most of the study at a prevalence > 67% and similar to the uro-fecal samples, there was also a decline in prevalence on day 29 p.i. (Table 3.1). Detection of *R. salmoninarum* in the mucus samples occurred at a more gradual rate than the other samples; however, at day 15 p.i., the prevalence increased substantially and remained consistent (Table 3.1).

Based on clinical signs and laboratory findings, the immersion-challenged fish exhibited an extremely mild course of a chronic, or sub-chronic, nature. On the contrary of the injection-challenged fish, characteristic clinical signs of BKD were seldom seen (Figure 3.2A-B). When present, disease signs included exophthalmia, sometimes associated with ocular hemorrhage (Figure 3.2A), diffuse petechial hemorrhages in the caudal peduncle region, occasionally associated with hemorrhages between the fin rays (Figure 3.2B), swollen spleens, generalized visceral edema, darkened kidneys, and swollen and pale livers. Additionally, there was one mortality that occurred on day 33 p.i. throughout the 105-day study period. The mortality exhibited the external and internal clinical signs described above. There were no clinical signs observed in the control (i.e., non-infected) fish.

Consequently, *R. salmoninarum* was detected at a lower prevalence in the lethal and non-lethal samples collected in the immersion challenge compared to the injection challenge

(Table 3.1). While the detection of *R. salmoninarum* progressively increased in the lethal samples at several of the sampling periods in the immersion challenge, there was more inconsistency in the detection of *R. salmoninarum* in the non-lethal samples (Table 3.1). *Renibacterium salmoninarum* was detected in the uro-fecal samples on days 1 and 63 p.i., in the mucus samples on day 21 p.i., and in the blood samples on days 1 and 84 p.i. (Table 3.1). However, it should be noted that *R. salmoninarum* was observed in the uro-fecal samples at much higher prevalences than it was detected in the blood and mucus samples.

4.3. Comparison of *R. salmoninarum* detection in all samples

In the injection-challenged fish, *R. salmoninarum* was detected at a prevalence of 100% in the lethal and the combined non-lethal samples, resulting in an AUC value of 1.00 for each, indicating that in a sub-acute infection, the collective non-lethal samples are equally effective as the lethal samples when detecting *R. salmoninarum* (Table 3.2). Individually, the uro-fecal and blood samples had 'excellent' discriminatory abilities to correctly identify the presence (or absence) of *R. salmoninarum* (Table 3.2). The respective sensitivity and accuracy for the mucus samples were low, resulting in a decreased, yet still 'acceptable,' AUC value (Table 3.2).

Similar to the injection challenge, the uro-fecal samples from the immersion challenge had the best success at detecting BKD, with the highest AUC value of all the non-lethal samples (Table 3.2). Infections in blood and mucus samples were detected at very low prevalences, yielding very low sensitivities and poor AUC values (Table 3.2). Conversely, *R. salmoninarum*

was detected more frequently in the lethal samples and resulted in 'excellent' discriminatory abilities (Table 3.2).

In a natural infection, it is more likely that fish will exhibit multiple stages of infection and a population will be comprised of fish in various stages of disease progression (Faisal and Eissa, 2009; Nance et al., 2010). Therefore, results from the injection and immersion challenge were combined to represent a population equally comprised of both disease stages (sub-acute and chronic). Based on these results, the sensitivities of the non-lethal samples (combined and individual) were distinctly lower the sensitivity of the lethal sample, suggesting that the non-lethal samples are not as effective at identifying positive samples as the lethal samples (Table 3.2). Due to the lower sensitivities, the accuracy of each tissue type was also lower than would be desired; however, the AUC value for the uro-fecal sample was in the middle of the 'acceptable' range (Table 3.2).

The specificity for all sample types was 1.00, implying that there were no false positives and that each sample type correctly identified the true negative samples 100% of the time. However, this is an artifact of the assumption that all infected fish were positive; therefore, specificity was chosen to not be reported in this study.

4.4. Comparison of diagnostic assays among sample types

Interestingly, there were clear differences in the prevalence, sensitivity, accuracy, and AUC values of the various samples when analyzed by the assay that was used (ELISA, nPCR, or culture). The prevalence of *R. salmoninarum* in the lethal samples was the highest when tested

by ELISA, followed by nPCR, and culture (Table 3.3). Furthermore, the lethal samples were in the highest agreement with the true positive status when tested by ELISA, with 'excellent' discrimination (Table 3.3). When all of the non-lethal samples were combined together, samples tested by nPCR yielded the highest prevalence; however, culture, ELISA, and nPCR all yielded identical accuracy, sensitivity, and AUC values (Table 3.3). Additionally, the AUC values indicate that they when any of the assays are used, they all have an 'acceptable' ability to correctly identify diseased and non-diseased individuals (Table 3.3). Similarly, a comparable AUC value can also be achieved if only the uro-fecal sample is collected and tested by nPCR (Table 3.3), which eliminates the need for the blood and mucus sample to also be analyzed. Interestingly, ELISA resulted in the highest accuracy, sensitivity, and AUC values for the blood and mucus samples (Table 3.3).

Based on the encouraging detection of *R. salmoninarum* in the uro-fecal samples in this study, when tested by all assays (culture, ELISA, and nPCR) and by nPCR alone, this sample was selected for further analysis to evaluate its potential use in conjunction with the traditional lethal sample. Specifically, when nPCR results from the uro-fecal sample and ELISA results from the lethal sample were combined, a relatively high level of agreement was observed (Table 3.4). In the injection challenge, *R. salmoninarum* was detected concurrently in both sample types in most of the fish (Table 3.4). In the immersion challenge, there was a greater disagreement between the lethal and uro-fecal sample, with a greater proportion of *R. salmoninarum* being detected in the lethal sample only (Table 3.4). Moreover, if all of the *R. salmoninarum*-positive lethal and uro-fecal samples were combined (L+/UF-, L-/UF+, L+/UF+), the overall detection of

R. salmoninarum increased substantially in the immersion challenge, from 69% in the lethal samples and 33% in the non-lethal samples to 81% overall (Table 3.4).

4.5. Population disease composition and estimated sampling size

While it is likely that fish will exhibit several disease stages in a naturally infected fish population, it is unlikely that the population will be comprised of equally distributed chronic and sub-acute infections. Therefore, when the sensitivity, accuracy, and AUC values were calculated for populations composed of varying infection levels, the results were more diverse (Table 3.5). If non-lethal samples were collected from a population of fish that was comprised of entirely sub-acute infections, the non-lethal samples would be highly sensitive and accurate, with perfect discrimination (Table 3.5). However, as the percentage of fish suffering from a chronic disease course progressively increases, the sensitivity, accuracy, and discriminatory abilities of the non-lethal samples decreases (Table 3.5). Once the population is comprised of 90% chronic infections, the discrimination of the non-lethal samples is no longer acceptable (Table 3.5). Similarly, if lethal samples were collected from a population of fish comprised of sub-acute infections, they would also be highly sensitive and accurate, with perfect discrimination (Table 3.5). However, as the percentage of fish suffering from a chronic disease course increases, the accuracy and sensitivity decline, but the discriminatory abilities are 'excellent,' even when the population consists of entirely chronic infections (Table 3.5). Most interestingly, if a uro-fecal sample and a lethal sample were collected from a similar population,

and tested by nPCR and ELISA, respectively, the highest accuracy, sensitivity, and AUC values were observed regardless of the disease composition of the population (Table 3.5).

The number of samples (e.g., lethal, combined non-lethal, and a combined uro-fecal and lethal sample) that would need to be collected to determine if BKD was present in a wild population was also estimated. If a population consisted of only sub-acute infections, it would require collecting lethal, non-lethal, and combined uro-fecal and lethal samples from 59 fish and 29 fish, with a MEP of 5% and 10%, respectively (Table 3.6). Furthermore, the number of samples required to be collected increased as the percentage of chronic infections increased, regardless of sample type or MEP (Table 3.6). However, noticeable differences were observed in the number of fish that would need to be sampled lethally and those that would have an additional uro-fecal sample collected (Table 3.6). Specifically, even though an additional sample is required, fewer fish would need to be tested if a lethal and uro-fecal sample were analyzed together compared to only a lethal sample (Table 3.6).

5. Discussion

Indeed, *R. salmoninarum* was detected in all of the non-lethally collected samples; however, differences were observed among sample types and sampling periods. *Renibacterium salmoninarum* was detected most frequently from the urine/feces mixture, followed by the blood, and lastly, the mucus. This is in agreement with other studies that have documented the presence of *R. salmoninarum* in fish intestines and feces (Bullock et al., 1978; Bullock et al., 1980; Mitchum and Sherman, 1981; Austin and Rayment, 1985; Balfry et al., 1996). Balfry et al.

(1996) detected *R. salmoninarum* in Chinook salmon feces and demonstrated fecal-oral transmission of the bacterium. It was suggested by Austin and Rayment (1985), who recovered *R. salmoninarum* from the feces of rainbow trout, that *R. salmoninarum* may have an affinity for organic, particulate matter. In this study, in 14 of the 16 fish where *R. salmoninarum* was concurrently isolated from both the uro-fecal sample and the kidney/spleen homogenate, the CFU g^{-1} of tissue was greater in the uro-fecal sample. In addition, there were seven samples where *R. salmoninarum* was recovered from the uro-fecal samples, but not the kidney/spleen homogenate. Interestingly, Mitchum and Sherman (1981) also found that *R. salmoninarum* was more readily detectable in the feces of several salmonids species compared to the kidneys.

Some researchers have attributed the presence of *R. salmoninarum* in the feces of infected salmonids to the accumulation of macrophages containing *R. salmoninarum* in the gut associated lymphoid tissue (GALT) in the intestine. *Renibacterium salmoninarum* has a predilection for the macrophages of the fish host, and has been shown to live and replicate within them (Gutenberger et al., 1997; Wiens and Kaattari, 1999). Therefore, the accumulation of macrophages containing *R. salmoninarum* in the GALT is a possible explanation for the high detection rate of *R. salmoninarum* in the uro-fecal samples. Also, since *R. salmoninarum* exists in the kidneys, some bacteria can travel in the urine outside of the fish host. In other words, the high prevalence of *R. salmoninarum* in uro-fecal samples is likely due to the shedding of the bacterium in both the digestive and urinary systems.

Taking into consideration that *R. salmoninarum* was detected in all of the uro-fecal samples on the first sampling period of the immersion challenge, yet there was a considerably lower prevalence in later sampling periods, it is also possible that *R. salmoninarum* utilizes the

anal opening as a portal of entry, which could have resulted in the higher initial prevalence of the bacterium. Also, once an infection is established, increased rates of *R. salmoninarum* being shed with the feces could lead to increased horizontal transmission and result in high prevalences of *R. salmoninarum*, as was seen in the i.p. injection challenge. In this context, the high prevalence of *R. salmoninarum* in the feces and resultant shedding could also contribute to horizontal transmission via a fish host ingesting the bacterium. Furthermore, as an infection progresses and the fish reduce their feeding, less fecal material would be available to shed the bacterium, resulting in a decline in the prevalence of *R. salmoninarum* in the feces, as was demonstrated on the last day of the injection challenge. Thus, findings from this study suggest that *R. salmoninarum* is readily detected in a urine/feces mixture from fish, which could help establish how frequently fish are being exposed to *R. salmoninarum*.

Even though the mucus sample was the least effective at detecting *R. salmoninarum* in this study, it is the first report of *R. salmoninarum* being recovered from the mucus layer of a fish. Hoffman et al. (1984) isolated *R. salmoninarum* from skin lesions from brown trout, coho salmon, and rainbow trout, but not from the actual mucus. Mucus is first and foremost a physical barrier to pathogens, as it is constantly being produced and sloughed from the surface, physically trapping and removing the bacteria before it can invade the fish's tissues (Ellis, 2001). Yet it also has a protective role in fish immunity and contains proteolytic enzymes, lymphocytes, antibodies, and lysozyme, which are important components of the fish immune response (Ourth, 1980; Hjelmeland et al., 1983; St. Louis-Cormier et al., 1984; Ellis, 2001). It was interesting to note, however, that the *R. salmoninarum* infection prevalence in mucus collected from immersion-challenged fish was much lower than that of injection-challenged

fish. One potential explanation is that injection-challenged fish consistently had a higher infection prevalence in their uro-fecal samples than did immersion-challenged fish, possibly indicating higher loads of bacteria being shed into the water that could result in more bacteria becoming imbedded within the mucus layer.

Renibacterium salmoninarum was also detected, albeit to a lesser degree, in the blood samples collected in this study. The level of prevalence depended on the exposure route, with a higher observed prevalence in injection infected fish. This finding lends support that a state of bacteremia is longstanding in the sub-acute course of the disease.

Despite the fact that fish in the immersion challenge were exposed to a higher total number of bacteria in the water compared to the injection challenge, injection-challenged fish developed a sub-acute course of BKD, exhibiting clear clinical signs, while the immersion-challenged fish exhibited a milder course of BKD, with very few clinical signs. Granulomatous-like lesions in the kidney were noticed in several fish from multiple sampling periods in the injection group, but were not observed in the immersion group. The differences noted in the immersion and i.p. injection challenges were most likely due to the nature of their exposure to the bacteria. Intraperitoneal injection delivers the bacteria directly into the fish, bypassing its external defense mechanisms; whereas an immersion challenge does not forego these important components of the immune system.

Clearly, the exposure route and resultant disease course impact the detection capabilities of lethal and non-lethal samples. *Renibacterium salmoninarum* was detected at much higher levels in the samples collected from fish exhibiting a sub-acute disease course than a chronic course. In the sub-acute fish, *R. salmoninarum* was able to spread throughout the fish

to all of the organs that were tested (kidney, spleen, intestine, blood, and mucus) and was detected more consistently. Conversely, the chronic nature of the immersion challenge impeded the ability to consistently detect *R. salmoninarum* from the non-lethally collected samples. In all three scenarios (sub-acute, chronic, or a combined infection), the uro-fecal sample was the ideal candidate for non-lethal disease detection.

While testing a lethal sample (i.e., kidney and spleen homogenate) is the current method to determine if a fish is infected (AFS-FHS, 2012; WOAH, 2012), there was some concern as to whether they reflected the true disease status and they were not chosen to be used as the 'gold standard' against which the non-lethal samples were compared. In both the immersion and i.p. injection challenge, fish were exposed to or injected with virulent *R. salmoninarum*; therefore, I contend that *R. salmoninarum* should have been detected in all of the lethal samples. In fact, on day 1 and 63 p.i. in the immersion challenge, there were 17 fish where *R. salmoninarum* was detected in the uro-fecal sample, but not the lethal sample; signifying that the lethal samples alone missed almost 10% of the positive fish. However, it is important to point out that the lethal samples had the best ability to determine true positive and negative samples, followed by the uro-fecal samples.

The assays used in this study (culture, ELISA, and nPCR) have been previously shown to yield variable results when used on the same sample (Cipriano et al., 1985; Pascho et al., 1987; White et al., 1995; Faisal and Eissa, 2009; Schulz, Chapter 2); therefore, how the diagnostic assay affected the efficacy of the non-lethal sample was evaluated. Interestingly, nPCR was determined to be the best assay to correctly identify positive and negative fish in the uro-fecal samples. The nPCR that was used in this study is highly specific for *R. salmoninarum*, and only

requires a small amount of DNA be present for detection (Chase and Pascho, 1998). The high detection of *R. salmoninarum* by nPCR in the uro-fecal samples could be due to a continuous shedding of the bacterium into the surrounding environment. Conversely, ELISA was better suited for detecting *R. salmoninarum* in the blood, mucus, and lethal samples than nPCR, which is one of the most widely used assays for detection of *R. salmoninarum* (Pascho and Mulcahy, 1987; Pascho et al., 1987; Pascho et al., 1991; White et al., 1995; Jansson et al., 1996; Bruno, 2004; Schulz, Chapter 2).

The detection rate of non-lethal samples was greatly enhanced by combining the collection and testing of both the lethal and uro-fecal samples tested by ELISA and nPCR, respectively. The lethal sample is comprised of a sample of the kidneys and spleen, organs which frequently remove circulating bacterial antigens from the host and provide an indication of a septicemic infection (Bruno and Poppe, 1996). Also, persistence of *R. salmoninarum* in the sediment and water could be reflected in the uro-fecal sample, indicating whether fish are actively shedding the bacterium and re-infecting other individuals. Therefore, collection of both a kidney/spleen homogenate and uro-fecal sample could shed light on the presence of *R. salmoninarum* in the infected host, as well as its presence in the surrounding environment. Additional knowledge regarding the extent of an *R. salmoninarum* infection in an aquaculture facility would assist biologists in making better management and treatment decisions. Moreover, with the current WOAH (2012) and AFS-FHS (2012) approved sampling methods, the only additional sample to be tested would be the uro-fecal sample, which is relatively inexpensive and easy to collect. Additionally, while nPCR would be an additional assay to perform, there are several well-defined conventional and nested PCR procedures in existence

(Brown et al., 1994; Chase and Pascho, 1998; Hong et al., 2002) that can be adapted and readily carried out in most laboratories.

While the combined non-lethal samples did not yield very high sensitivities from immersion-challenged fish, it was still of interest to evaluate their potential use in a wild population. As mentioned previously, the effectiveness of the non-lethal samples depended on the disease course of infected individuals. It is difficult to assess disease course in wild populations, although it is most likely that the wild fish populations will have multiple disease courses occurring simultaneously (Faisal and Eissa, 2009; Nance et al., 2010; Elliott et al., 2013), therefore the combined results from the injection and immersion challenge are the most relevant for a wild population. Interestingly, the most promising results indicated that collecting a lethal *and* uro-fecal sample could result in fewer fish being required for testing as a population consisted of more chronic infections. The addition of the uro-fecal sample resulted in higher diagnostic sensitivities, increasing the likelihood of detecting more true positives and less false negatives. As such, it is suggested that a urine/feces mixture should be collected in addition to the traditional kidney/spleen sample to improve the overall detection of *R. salmoninarum* and potentially reduce the number of fish needed for sampling.

Non-lethal sampling may still be a viable alternative in certain scenarios. For example, the Endangered Species Act of 1973 prohibits harmful actions to any endangered or threatened animal species, including species that need to undergo disease testing. It is recommended by the United States Fish and Wildlife Service that when lethal sampling of endangered and threatened species is prohibited, non-lethal sampling techniques should be considered (Heil, 2004). Similar to endangered species, biologists and managers may not be able to sacrifice

valuable broodstock populations for lethal disease testing either. In these situations, even though more fish may need to be sampled with lesser diagnostic sensitivity, it may be a viable alternative.

APPENDIX

Table 3.1. The prevalence of *Renibacterium salmoninarum* (with the number of positive fish) detected from the lethally (kidney and spleen homogenate) and non-lethally (combined, uro-fecal, blood, and mucus) collected samples from each sampling period (days post-infection) and overall ('total') during the injection challenge, immersion challenge, and a combination of the injection and immersion challenge.

Challenge	Days post-infection	Prevalence				
		Lethal	Combined non-lethal	Uro-fecal	Blood	Mucus
Injection	1	100% (15)	100% (15)	100% (15)	100% (15)	0% (0)
	8	100% (15)	100% (15)	100% (15)	73% (11)	13% (2)
	15	100% (15)	100% (15)	100% (15)	93% (14)	87% (13)
	22	100% (15)	100% (15)	100% (15)	73% (11)	100% (15)
	29	100% (3)	100% (3)	33% (1)	67% (2)	100% (3)
	Total	100% (63)	100% (63)	97% (61)	84% (53)	52% (33)
Immersion	1	24% (5)	100% (21)	100% (21)	5% (1)	0% (0)
	21	62% (13)	24% (5)	0% (0)	0% (0)	24% (5)
	42	76% (16)	0% (0)	0% (0)	0% (0)	0% (0)
	63	91% (19)	52% (11)	52% (11)	0% (0)	0% (0)
	84	62% (13)	5% (1)	0% (0)	5% (1)	0% (0)
	105	93% (13)	0% (0)	0% (0)	0% (0)	0% (0)
	Total	69% (79)	33% (38)	28% (32)	2% (2)	4% (5)
Combined	Total	80% (142)	57% (101)	52% (93)	31% (55)	21% (38)

Table 3.2. Diagnostic performance of the lethal and non-lethal samples (uro-fecal, blood, and mucus) from the injection and immersion challenges, and a combination of the challenges. The accuracy and sensitivity (with 95% confidence intervals), and the area under the curve (AUC) values are reported. *AUC values that have acceptable (0.7-0.8), excellent (0.8-0.9), outstanding (0.91-0.99), or perfect (1.00) discrimination. AUC values < 0.6 are no better than chance alone.

Challenge	Sample	Accuracy	Sensitivity	AUC
Injection	Lethal	1.00 (0.96-1.00)	1.00 (0.94-1.00)	1.00*
	Non-lethal	1.00 (0.96-1.00)	1.00 (0.94-1.00)	1.00*
	Uro-fecal	0.98 (0.92-1.00)	0.97 (0.89-1.00)	0.99*
	Blood	0.89 (0.81-0.95)	0.84 (0.73-0.92)	0.92*
	Mucus	0.67 (0.57-0.77)	0.52 (0.39-0.65)	0.76*
Immersion	Lethal	0.72 (0.64-0.79)	0.65 (0.56-0.74)	0.83*
	Non-lethal	0.48 (0.40-0.57)	0.33 (0.25-0.42)	0.67
	Uro-fecal	0.44 (0.36-0.53)	0.28 (0.20-0.37)	0.64
	Blood	0.24 (0.18-0.32)	0.02 (0.002-0.06)	0.51
	Mucus	0.26 (0.19-0.34)	0.04 (0.01-0.10)	0.52
Combined	Lethal	0.82 (0.77-0.87)	0.77 (0.70-0.83)	0.89*
	Non-lethal	0.67 (0.61-0.73)	0.57 (0.49-0.64)	0.79*
	Uro-fecal	0.64 (0.58-0.70)	0.52 (0.45-0.60)	0.76*
	Blood	0.48 (0.41-0.54)	0.31 (0.24-0.38)	0.66
	Mucus	0.40 (0.34-0.47)	0.21 (0.16-0.28)	0.61

Table 3.3. Diagnostic performance of all of the lethally and non-lethally (uro-fecal, blood, and mucus) collected samples from the combined injection and immersion challenges. The prevalence (with the number of positive fish), accuracy and sensitivity (with 95% confidence intervals), and the area under the curve (AUC) values are reported as detected by bacterial culture, nested polymerase chain reaction (PCR), semi-quantitative enzyme-linked immunosorbent assay (ELISA), and by all assays combined. *AUC values that have acceptable (0.7-0.8) or excellent (0.8-0.9) discrimination. AUC values < 0.6 are no better than chance alone.

Sample Type	Assay	Prevalence	Accuracy	Sensitivity	AUC
Non-lethal	Combined	57% (101)	0.44 (0.38-0.50)	0.51 (0.43-0.58)	0.63
	ELISA	35% (63)	0.67 (0.61-0.73)	0.57 (0.49-0.64)	0.79*
	nPCR	53% (95)	0.67 (0.61-0.73)	0.57 (0.49-0.64)	0.79*
	Culture	31% (56)	0.67 (0.61-0.73)	0.57 (0.49-0.64)	0.79*
Uro-fecal	Combined	52% (93)	0.37 (0.30-0.43)	0.34 (0.27-0.42)	0.61
	ELISA	29% (52)	0.46 (0.40-0.53)	0.29 (0.23-0.36)	0.65
	nPCR	52% (93)	0.64 (0.57-0.70)	0.52 (0.44-0.60)	0.76*
	Culture	13% (23)	0.34 (0.28-0.41)	0.13 (0.08-0.19)	0.57
Blood	Combined	31% (55)	0.31 (0.25-0.37)	0.20 (0.14-0.26)	0.43
	ELISA	25% (45)	0.35 (0.29-0.42)	0.15 (0.10-0.21)	0.58
	nPCR	11% (20)	0.33 (0.27-0.39)	0.11 (0.07-0.17)	0.56
	Culture	28% (49)	0.45 (0.39-0.52)	0.28 (0.21-0.35)	0.64
Mucus	Combined	21% (38)	0.29 (0.24-0.36)	0.14 (0.09-0.20)	0.46
	ELISA	15% (26)	0.43 (0.37-0.50)	0.25 (0.19-0.32)	0.63
	nPCR	16% (28)	0.36 (0.30-0.43)	0.15 (0.11-0.22)	0.58
	Culture	6% (10)	0.29 (0.23-0.35)	0.06 (0.03-0.10)	0.53
Lethal	Combined	80% (142)	0.50 (0.43-0.56)	0.57 (0.49-0.64)	0.43
	ELISA	79% (140)	0.84 (0.79-0.88)	0.79 (0.72-0.84)	0.90*
	nPCR	34% (61)	0.50 (0.44-0.57)	0.34 (0.27-0.42)	0.67
	Culture	21% (38)	0.40 (0.34-0.47)	0.21 (0.16-0.28)	0.61

Table 3.4. The prevalence of *Renibacterium salmoninarum* (with the number of positive fish) detected from lethal (kidney and spleen homogenate) samples only (L+/UF-), uro-fecal samples only (L-/UF+), and both lethal and uro-fecal samples (L+/UF+) using results from ELISA for the lethal samples and from nPCR for the uro-fecal samples. Samples were collected from the injection challenge, immersion challenge, and a combination of the injection and immersion challenge.

Challenge	L+/UF-	L-/UF+	L+/UF+	Total
Injection	3% (2)	0%	97% (61)	100% (63)
Immersion	53% (61)	15% (17)	13% (15)	81% (93)
Combined	35% (63)	10% (17)	43% (76)	88% (156)

Table 3.5. Diagnostic performance of lethally collected samples (LETH), non-lethally collected samples (NON), and a combined uro-fecal and kidney/spleen sample (UF/KS) from the combined injection (sub-acute) and immersion (chronic) challenges at varying percentages in a fish population. Lethal and non-lethal samples were tested by all assays (bacterial culture, nested polymerase chain reaction (nPCR), and the semi-quantitative enzyme-linked immunosorbent assay (ELISA), while the uro-fecal and kidney/spleen sample was tested by nPCR and ELISA, respectively. The accuracy, sensitivity, and area under the curve (AUC) values are reported. *AUC values of acceptable (0.7-0.8), excellent (0.8-0.9), outstanding (0.91-0.99), or perfect (1.00) discrimination. AUC values < 0.6 are no better than chance alone.

Sub-acute	Chronic	Accuracy			Sensitivity			AUC		
%	%	LETH	NON	UF/KS	LETH	NON	UF/KS	LETH	NON	UF/KS
100	100	1.00	0.99	1.00	1.00	0.99	1.00	1.00*	1.00*	1.00*
90	10	0.96	0.91	0.98	0.95	0.89	0.97	0.98*	0.95*	0.99*
80	20	0.93	0.84	0.96	0.90	0.79	0.94	0.95*	0.90*	0.97*
70	30	0.90	0.78	0.95	0.87	0.70	0.93	0.94*	0.85*	0.97*
60	40	0.87	0.72	0.92	0.83	0.63	0.90	0.92*	0.82*	0.95*
50	50	0.85	0.67	0.91	0.80	0.57	0.89	0.90*	0.79*	0.95*
40	60	0.82	0.62	0.90	0.77	0.50	0.86	0.89*	0.75*	0.93*
30	70	0.81	0.59	0.89	0.75	0.46	0.85	0.88*	0.73*	0.93*
20	80	0.79	0.55	0.88	0.73	0.41	0.84	0.87*	0.71*	0.92*
10	90	0.77	0.51	0.87	0.71	0.36	0.83	0.86*	0.69	0.92*
0	100	0.76	0.48	0.86	0.69	0.33	0.82	0.85*	0.67	0.91*

Table 3.6. The estimated samples size needed for testing to conclude with 95% confidence that disease is not present in a population of 10,000 fish was calculated at a minimum expected prevalence (MEP) of 5 and 10% for lethally collected samples (LETH), non-lethally collected samples (NON), and a combined uro-fecal and kidney/spleen sample (UF/KS) from the combined injection (sub-acute) and immersion (chronic) challenges at varying percentages in a fish population. Lethal and non-lethal samples were tested by all assays (bacterial culture, nested polymerase chain reaction (nPCR), and the semi-quantitative enzyme-linked immunosorbent assay (ELISA), while the uro-fecal and kidney/spleen sample was tested by nPCR and ELISA, respectively.

Sub-acute	Chronic	5% MEP			10% MEP		
%	%	LETH	NON	UF/KS	LETH	NON	UF/KS
100	100	59	59	59	29	29	29
90	10	62	66	61	30	33	30
80	20	66	75	63	32	37	31
70	30	68	85	63	33	42	31
60	40	71	94	66	35	47	32
50	50	74	104	66	36	52	33
40	60	77	119	69	38	59	34
30	70	79	129	69	39	64	34
20	80	81	145	70	40	72	35
10	90	83	165	71	41	80	35
0	100	86	120	72	42	90	35

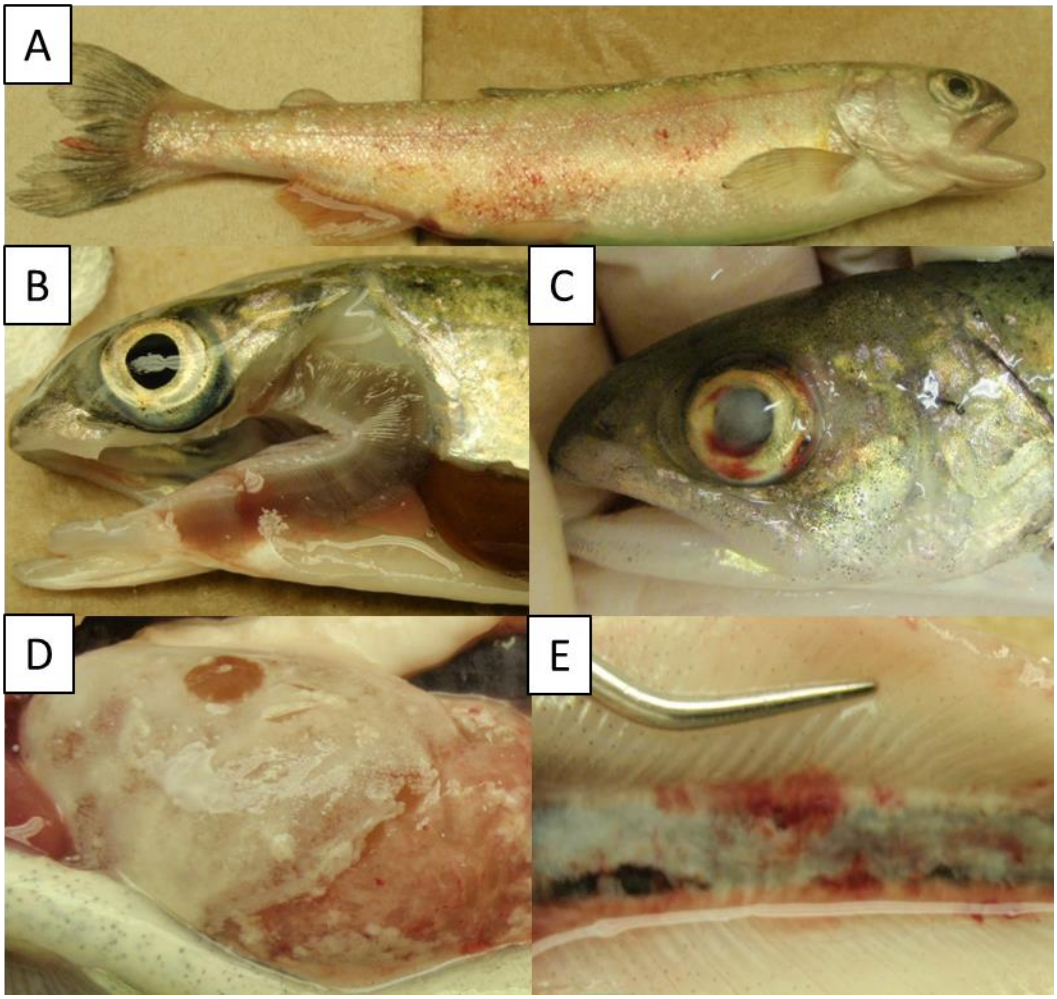

Figure 3.1. Characteristic bacterial kidney disease clinical signs observed in the injection challenge included (A) petechial hemorrhage throughout the body of the fish (B) gill pallor, (C) corneal opacity with associated hemorrhage, (D) false membranes overlying the liver, and (E) granulomatous-like lesions in the kidney.

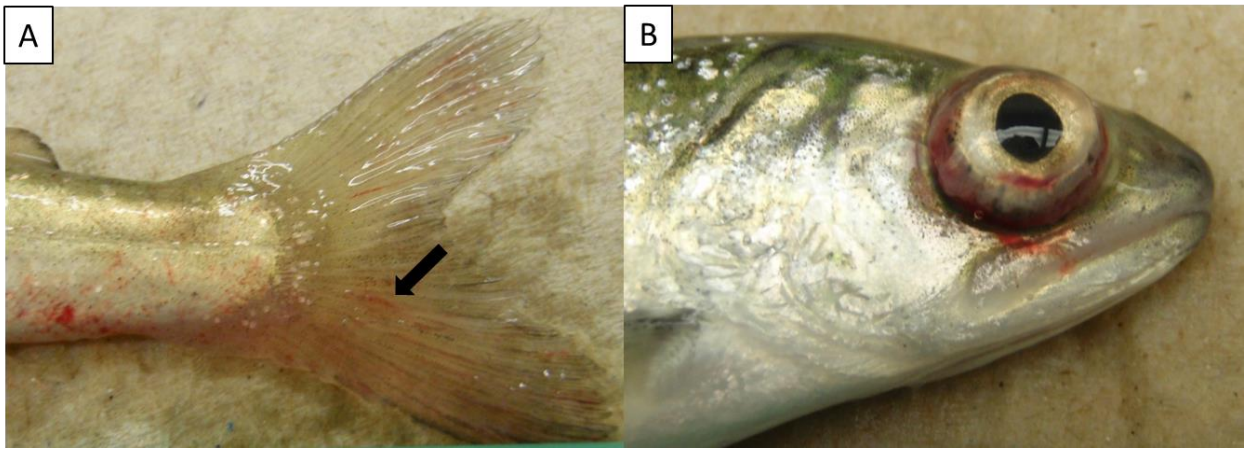

Figure 3.2. Characteristic bacterial kidney disease clinical signs observed in the immersion challenge included (A) petechial hemorrhages in the caudal peduncle region, as well as between the fin rays (black arrow), and (B) exophthalmia and associated hemorrhage.

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Chapter 4

Evidence of anti-*Renibacterium salmoninarum* Antibodies in Naturally and Experimentally Infected *Oncorhynchus* spp.

1. Abstract

Bacterial kidney disease, caused by *Renibacterium salmoninarum*, is a long-standing problem among wild and aqua-cultured salmonids in the Great Lakes basin and Pacific Northwest. Several assays have been developed for the detection of *R. salmoninarum* in fish tissues, including the enzyme-linked immunosorbent assay (ELISA). In this study, a single-dilution indirect-ELISA was assessed with the aim to detect anti-*R. salmoninarum* antibodies in experimentally infected juvenile rainbow trout (*Oncorhynchus mykiss*) and adult Chinook salmon (*O. tshawytscha*), coho salmon (*O. kisutch*), and steelhead. Over a 26 wk period, antibody levels were measured every 3 wks in multiple groups of naïve (n=169) and non-naïve (n=171) rainbow trout that were injected intraperitoneally (i.p.) with live *R. salmoninarum*. Antibody production and mean time-to-death was determined over a 33 wk period in an additional group of i.p. injected naïve rainbow trout (n=46). Lastly, feral Chinook salmon (n=60), coho salmon (n=60), and steelhead (n=40) returning to spawn at Lake Michigan weirs were also assessed for production of anti-*R. salmoninarum* antibodies. The intensity of the infection in the kidneys and spleen in all fish groups was detected using standard bacterial culture techniques and a sandwich ELISA. All groups of experimentally infected and feral fish were found to produce detectable levels of antibodies, although there were minimal differences among them. The observed antibody response in experimentally infected fish appeared to be short-lived and long-term elevated levels of antibody production were not observed. Despite low infections of *R. salmoninarum* in the kidneys and spleen, feral fish still produced elevated levels of antibodies, suggesting that despite a currently low prevalence of BKD in the Great

Lakes basin, exposure to *R. salmoninarum* is occurring and resulting in the production of circulating antibodies.

2. Introduction

Significant losses associated with *Renibacterium salmoninarum*, the causative agent of bacterial kidney disease (BKD), have been documented in infected salmonids from both marine (Ellis et al., 1978; Paterson et al., 1979; Banner et al., 1983; Meyer et al., 1999) and freshwater systems (MacLean and Yoder, 1970; Mitchum et al., 1979; Schulz, Chapter 2) worldwide. In particular, BKD poses a significant risk for wild and aqua-cultured salmonids in the Great Lakes basin (GLB) and Pacific Northwest (PNW), where the pathogen is persistent, often causing clinical episodes (Mitchum and Sherman, 1981; Hnath and Zischke, 1991; Maule et al., 1996; Schulz, Chapter 2). Bacterial kidney disease is particularly difficult to control due to its ability to be transmitted by both horizontal and vertical routes, thus routine health monitoring of broodstock and their offspring, with culling of infected fish, is one of the most important methods for combatting BKD (Schulz, Chapter 2).

Several assays have been developed for the detection of *R. salmoninarum* in fish tissues, eggs, and ovarian fluid by bacterial culture (Evelyn, 1977; Austin et al., 1983; Faisal et al., 2010a), molecular assays (Brown et al., 1994; Chase and Pascho, 1998; Chase et al., 2006), and immunological methods such as Western blot, immunofluorescence techniques, and sandwich enzyme-linked immunosorbent assay (ELISA) (Pascho and Mulcahy, 1987; Griffiths et al., 1991; Hsu et al., 1991; Jansson et al., 1996). On the contrary, the detection and quantitation of

circulating antibodies against *R. salmoninarum* has received little attention, and has been, for the most part, based on experimental infections. For example, Evelyn (1971) developed an agglutination assay to determine antibody titers in experimentally infected sockeye salmon (*Oncorhynchus nerka*). However, it was not determined if the detected antibodies conferred any protective immunity in this experiment. Later, Olivier et al. (1992) used Western blot to monitor the production of antibodies against *R. salmoninarum* in experimentally infected Atlantic salmon.

With the expansion of monoclonal antibody production, several ELISA based assays have been developed to detect whole *R. salmoninarum* bacteria, its soluble antigen, or specific anti-*R. salmoninarum* antibodies circulating in sera of surviving fish (Pascho and Mulcahy, 1987; Bartholomew et al., 1991; Hsu et al., 1991; Rockey et al., 1991). Other than experimentally-infected fish, antibody assessment in cultured, feral, or wild salmonids has seldom been used. However, Jansson and Ljungberg (1998) did detect an elevated antibody response to *R. salmoninarum* in farm-raised Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhynchus mykiss*) using an indirect ELISA, leading the authors to suggest this method may be valuable for monitoring stocks of fish that are known to have likely been exposed to *R. salmoninarum* or survived a BKD episode.

Using an ELISA-based assay to assess production of *R. salmoninarum* antibodies has gone through several phases of development. To semi-quantitate levels of antibodies being produced in a fish with ELISA, several dilutions of each serum sample are usually analyzed to calculate units of antibody or to arrive at an endpoint dilution (Wood and Kaattari, 1996). However, this method can increase the cost of the reagents and limit the number of fish

assessed on each ELISA run (Alcorn and Pascho, 2000). Therefore, a single-dilution antibody ELISA was developed with the aim to increase the number of samples being tested on each ELISA microplate, thereby decreasing the cost per sample (Alcorn and Pascho, 2000). The method required the establishment of a standard curve with a positive serum sample of known titer to function as a reference for the test samples (Alcorn and Pascho, 2000). Additionally, typical antibody quantitation by ELISA requires antibodies from the test sera to bind to an antigen that has been coated to the microplate wells for several hours before running the assay (Arkoosh and Kaattari, 1990). The main soluble antigen from *R. salmoninarum* consists primarily of a highly glycosylated protein with a molecular weight of ~57 kilodaltons (kDa), known as p57 (Getchell et al., 1985). Coating the wells with the overwhelmingly glycosylated p57 only can mask the detection and quantitation of fish antibodies directed against the bacteria themselves, and not their soluble antigen. This led Wood and Kaattari (1996) to develop an ELISA protocol for antibody detection with whole *R. salmoninarum* bacteria to coat the wells, which has lowered the costs associated with soluble antigen preparation.

The GLB has been plagued with *Renibacterium salmoninarum* since the 1950s with serious mortality of hatchery-raised salmonids frequently observed, a matter that necessitated the implementation of stringent control measures including broodstock culling, egg disinfection, and regular screening (Hnath, 1993). It is clear that the measures 7implemented basinwide have caused a dramatic reduction in the prevalence and intensity of *R. salmoninarum* infections (Schulz, Chapter 2) to the extent that BKD has become an endemic disease of less concern in Great Lakes (GL) *Oncorhynchus* species. While the reason for *R. salmoninarum* decline has been mainly attributed to the stringent biosecurity measures and

egg disinfection program, others have demonstrated that Chinook salmon from the Great Lakes are less susceptible and have decreased BKD-associated mortality upon experimental infection compared to their species and age cohort from the PNW (Purcell et al., 2008). The previous authors attributed this to pathogen-driven selection; when the GL Chinook salmon population was subjected to the mass fish mortalities in the late 1980s, the most resistant fish survived, resulting in a more resistant population overall (Purcell et al., 2008). However, it was unclear if the decline in BKD incidence in *Oncorhynchus* spp. is due to inherent resistance, or that the pathogen presence in the GLB has substantially diminished. The study performed by Faisal et al. (2010b), however, demonstrated that *R. salmoninarum* continues to be a threat to GL salmonids. The authors found high prevalence (~65%) and intensity of *R. salmoninarum* infection, as well as clinical BKD signs, in four naturally occurring (i.e., no artificial propagation and stocking) lake whitefish stocks in lakes Michigan and Huron. While the data of Faisal et al. (2010b) confirmed the continuous presence of *R. salmoninarum* in the Great Lakes watershed at levels sufficient to cause BKD in susceptible salmonids, it also raised the important question as to why wild *Oncorhynchus* species, sharing the same area with heavily infected lake whitefish, have not succumbed again to *R. salmoninarum* and developed clinical BKD.

To this end, this study was initiated to develop an ELISA-based assay that is capable of detecting and quantitating circulating antibodies against *R. salmoninarum* in experimentally infected juvenile fish, as well as adult *Oncorhynchus* spp. returning to spawn at gamete-collecting facilities. The modified ELISA assay developed in the course of this study enabled the assessment of the serological status in *Oncorhynchus* species exposed to *R. salmoninarum* under both laboratory and natural field conditions. Additionally, it was also tested if the

presence of circulating anti-*R. salmoninarum* antibodies can confer protection to fish experimentally exposed to this serious pathogen.

3. Materials and Methods

3.1. Determination of anti-*R. salmoninarum* antibodies in experimentally infected rainbow trout

Two groups of rainbow trout, acquired from Troutlodge, Inc. (Sumner, WA), were used in this study: 1) a group of rainbow trout (~11 months old; n=169) that survived a previous exposure to *R. salmoninarum* ('survivor'), and 2) a group of naïve rainbow trout (~8 months old; n=171) (Table 4.1). The survivor fish group (SF) acclimated to laboratory conditions for seven weeks, and was then exposed to a low dose of *R. salmoninarum* [6.6×10^5 colony forming units (CFU)] via treated food pellets for one week. Nine weeks later, survivors from this challenge were further divided into two subgroups: a group of rainbow trout which were immunized with an oral BKD bacterin (SFi) at a dose of 0.38 µg of antigen $fish^{-1}$ day^{-1}, and a group of non-immunized rainbow trout (SFn) that received sterile saline. This was followed by a second bacterin that was administered 16 weeks after the initial bacterin at a dose of 0.53 µg of antigen $fish^{-1}$ day^{-1}. Two weeks after administration of the secondary bacterin, fish were intraperitoneally (i.p.) injected with *R. salmoninarum* as described below.

Conversely, the naïve rainbow trout group (NF) acclimated to laboratory conditions for three weeks prior to being divided into similar subgroups. A portion of the NF fish group were

exposed to the same BKD oral bacterin at a dose of 0.085 µg of antigen $fish^{-1}$ day^{-1} (NFi), while a non-immunized group received sterile saline (NFn). Sixteen weeks later, the NFi group received a second bacterin dose of 0.16 µg of antigen $fish^{-1}$ day^{-1}. Similarly, two weeks post-secondary bacterin, fish were i.p. injected with *R. salmoninarum* as described below.

Prior to experimental challenge, *R. salmoninarum* (American Type Culture Collection #33209) was passed once through naïve rainbow trout to ensure infectivity. The recovered *R. salmoninarum* isolate was then inoculated onto modified kidney disease medium (MKDM; Faisal et al., 2010a), incubated at 15°C for 14 d, purity verified, and then a single colony was inoculated into a 7-ml aliquot of MKDM broth (x2) and incubated at 15°C for 7 d. Ten µl from both of the broth cultures was then sub-cultured onto trypticase soy agar (TSA) and MKDM to verify purity, and the remaining broth was added to 1000 ml of fresh MKDM broth and incubated at 15°C on a Thermolyne Nuova stir plate (Thermo Fischer Scientific, Inc., Waltham, MA) at approximately 50 rpm in a 2 L Celstir Spinner Flask (Wheaton, Millville, NJ). After 14 days of incubation, the broth culture was centrifuged in a Hermle Z382K centrifuge (Labnet International, Inc., Woodbridge, NJ) at 4300 rpm for 10 min and the supernatant was discarded. The bacterial pellet was re-suspended in 0.85% saline solution, centrifuged as described above, and the supernatant was discarded. This was repeated three times. The remaining pellet was re-suspended into 926 ml of sterile saline, for a final concentration of 3.7×10^{10} cfu ml^{-1}, which was used for i.p. injection.

Fish were first anesthetized with 100 mg/L of sodium bicarbonate-buffered tricaine methanesulfonate (MS-222; Argent Chemical Laboratories, Inc., Redmond, Washington) for approximately 10-15 sec, and were then i.p. injected with the bacterial suspension. Due to the

larger size of the SF group, survivor fish received 200 µl of the bacterial suspension, which is double the volume that was administered to the NF group (i.e, 100 µl). Infected NF and SF were then revived by placing them in freshwater in their 225 L and 74L tanks, respectively, and maintained at a water temperature of 12 ± 2.0°C. Triplicate tanks containing 26-27 fish per tank were used for each of the four fish groups. This was done in accordance with the Michigan State University International Animal Care and Use Committee (Animal Use Form #07/12-133-00). All tanks were monitored daily for mortalities.

3.2. Protection associated with anti-*R. salmoninarum* antibodies

To determine if circulating antibodies confer protection to rainbow trout, an additional experiment was designed with naïve rainbow trout (11 months old; n=46) divided into two subgroups: naïve rainbow trout immunized with the oral BKD bacterin (NPi) and a second group that was not immunized (NPn). The NPi group was exposed to a single dose of the BKD oral bacterin (4.9 µg of antigen $fish^{-1}$ day^{-1}) 29 weeks post-acclimatization, while the NPn group received sterile saline. Four weeks after bacterin administration, both groups were i.p. injected with 500 µl of a 3.7 x 10^{10} cfu ml^{-1} *R. salmoninarum* bacterial suspension, as described previously. Fish were then revived by placing them in freshwater in their respective 74 L tanks, with a water temperature of 12 ± 2.0°C. Five replicate tanks containing 4-5 fish per tank were used for each group. This was also done in accordance with the Michigan State University International Animal Care and Use Committee (Animal Use Form #07/12-133-00). All tanks

were monitored daily for mortalities. For each mortality, time to death (TTD) was recorded and samples were collected as described below.

3.3. Sample collection from experimentally infected fish

Samples were collected every three weeks post-infection (p.i.) from the SFi, SFn, NFi, and NFn groups (n=5), while samples were only collected from moribund or freshly dead NPi and NPn fish. Fish were euthanized with a lethal dose of 250 mg/L of sodium bicarbonate-buffered MS-222 (Argent) prior to sample collection. Once euthanized, individual lengths and weights were recorded and a thorough internal and external examination for gross signs of disease was performed. Blood samples were collected by venipuncture of the caudal vein using a sterile needle and syringe and were allowed to clot overnight at 4°C. Blood samples were then centrifuged in a Denville 300D Centrifuge at 5000 rpm for 10 min to separate the serum from the plasma, the serum was removed, aliquoted, and stored at -80°C until diagnostic assays were performed. Isolation of *R. salmoninarum* from the kidney was performed by streaking 10 µl of the kidney tissue onto MKDM agar plates using sterile disposable inoculating loops, and incubating the plates under aerobic conditions at 15°C for a total of 42 days. The remaining kidney/spleen sample was collected with individual sterile forceps and scissors, placed in individual whirlpaks, and stored at -80°C until the sandwich enzyme-linked immunosorbent assay (ELISA) was performed. Kidney/spleen tissue processing and ELISA were performed as described in Chapter 2.

3.4. Assessment of anti-*R. salmoninarum* antibodies in three *Oncorhynchus* spp. returning to spawn at gamete collecting facilities

During April, September, and October of 2009 and 2013, adult Chinook salmon (5.6 kg ± 2.5 kg), coho salmon (0.7 kg ± 2.6 kg), and steelhead (2.7 kg ± 5.6 kg) were collected from two Michigan Department of Natural Resources gamete-collecting weirs: Chinook salmon and steelhead from the Little Manistee River Weir (LMRW) and coho salmon from the Platte River Weir (PRW) (Figure 4.1; Table 4.1). Prior to sample collection, fish were euthanized by immersion in CO_2-laden water and manual blunt force trauma to the cranium. Blood was collected from the dorsal aorta at the caudal peduncle region and allowed to clot in sterile tubes overnight. Serum was separated as described above. Length, weight, and sex of all fish were recorded and external examinations were performed. Fish were externally disinfected with 70% ethanol and subjected to thorough internal clinical examinations. A kidney/spleen sample was collected as described above for ELISA analysis.

3.5. Modified single dilution indirect ELISA to detect and quantitate *R. salmoninarum* antibodies in fish sera

The *R. salmoninarum*-specific antibody response of experimentally and naturally infected fish was quantitated by an indirect ELISA based on Wood and Kaattari (1996) and Alcorn and Pascho (2000), with modifications. Briefly, the surfaces of a microplate were coated with formalin-killed *R. salmoninarum* cells (500 µg ml^{-1}) and were incubated at 17°C overnight.

Plates were then blocked with phosphate buffered saline (PBS) containing 1% goat serum (Sigma-Aldrich) for 1 hr at room temperature (RT). Test sera was diluted 1:10 in PBS containing 0.05% tween 20 (PBS-T20), added to the microplate, and incubated at RT for 2 hr. To help reduce non-specific binding, plates were washed three times with PBS-T20, followed by soaking the plates with PBS-T20 for three 10 min intervals. The presence of anti-*R. salmoninarum* activity was then detected using a purified anti-rainbow trout monoclonal antibody (CLF001AP, Cedarlane Labs, Burlington, NC) diluted 1:200 in PBS-T20 and was incubated at RT for 2 hr. After washing, an anti-mouse IgG-alkaline phosphatase antibody (Sigma-Aldrich), diluted 1:1000 in PBS-T20 containing 0.5% goat serum, was added and incubated at 37°C for 1 hr. Plates were washed again as previously described. Lastly, alkaline phosphatase substrate (Sigma-Aldrich) was added and the microplate was incubated in the dark at RT for 45 min. The plate was then read at 405 nm with a BioTek ELx808 (BioTek Instruments, Inc., Winooski, VT) plate reader.

Each ELISA run included a previously recognized high titer serum sample diluted two-fold (1:10, 1:20, 1:40, 1:80) and a diluted negative serum sample (1:10). The positive-negative cut-off absorbance for the samples was the mean anti-*R. salmoninarum* antibody activity (plus two standard deviations) of 4 month old naïve rainbow trout (n=20) with no history of exposure to *Renibacterium salmoninarum*, as recommended by Alcorn and Pascho (2000).

3.6. Statistical analyses

Comparisons of antibody production among the fish groups were done using a one-way ANOVA and assessed using SAS statistical software, version 9.3 (SAS Institute Inc., Cary, NC). Survival statistics were calculated by the Kaplan-Meier analysis also using SAS 9.3. Differences between the probability of survival of NPn and NPi fish were determined by the logrank comparison, with statistical significance set at $P \leq 0.05$.

4. Results

4.1. Experimental infection

Infection by *R. salmoninarum* and the development of BKD signs were observed in all four groups of the experimentally infected fish during the multiple sampling periods post-infection. Specifically, the intensity of the *R. salmoninarum* isolated from the kidneys (i.e., average CFUs) in the NFn and NFi groups peaked at week 3 p.i. and then steadily declined in subsequent sampling periods, until the infection disappeared by week 15 p.i. (Figure 4.2). The p57 antigen was detected in the kidneys and spleen by sandwich ELISA in all fish and continued its presence through the end of the observation period at week 26 p.i. (Figure 4.2). No trends were observed regarding the intensity of p57 between the two fish groups and among the sampling periods. As depicted in Figure 4.3, a similar trend was noticed in the levels of p57 antigen, as well as the number of *R. salmoninarum* CFUs isolated from the kidneys, in SFn and

SFi groups. The maximum intensity of infection levels in the SFn and SFi groups, however, were considerably lower (e.g., 67 CFUs) than the NFn and NFi groups (e.g., 101 CFUs) (Figure 4.3).

4.2. Antibody production in experimentally infected fish

Anti-*R. salmoninarum* antibodies were clearly detected in all four groups of experimentally infected fish, with a trend of increasing in levels starting as early as week 3 p.i. (Figures 4.4 and 4.5). Levels of antibodies averaged an OD value of 0.257 for all fish and reached a maximum OD value of 2.514. There were no clear differences among the production of antibodies in the four fish groups and the small sample size at each sampling period p.i. did not allow the performance of statistical analysis. However, the experiment was terminated at week 26 p.i., thus increasing the sample size, allowing for statistical analysis to be performed. Analysis showed that the NFn group had a significantly higher antibody response than the NFi group ($F = 6.09$; $df = 1, 88$; $P = 0.0388$), SFn group ($F = 10.25$; $df = 1, 85$; $P = 0.0126$), and SFi group ($F = 6.90$; $df = 1, 87$; $P = 0.0303$) at week 26 p.i.

Measurable antibodies were first observed in the NFn and NFi groups at week 6 p.i. and peaked at week 15 p.i. (Figure 4.4). After week 15 p.i., the production of antibodies started to decline in both groups, which was consistent with the rate of infection observed in the kidneys and spleen (Figure 4.2). Antibodies were detected as early as week 3 p.i. in the SFn group of fish, while the antibody response of the SFi group (OD = 0.0916) was just below the positive-negative threshold (OD = 0.094) (Figure 4.5). The antibody response of the SFn group peaked between weeks 9 and 12 p.i. (Figure 4.5), which is also the period that followed their highest

rates of infection (Figure 4.3). Similarly, the time period (weeks 12 to 24 p.i.) when the antibody response of the SFi group was highest also coincided with the most consistent infection rates, as demonstrated by the prevalence of the *R. salmoninarum* p57 antigen in the kidneys (Figure 4.5). Additionally, the SFi group of fish produced elevated anti-*R. salmoninarum* antibodies well into weeks 18 and 24 p.i., contrary to the other three experimental groups (Figure 4.5).

4.3. Detection of antibodies in adult *Oncorhynchus* spp.

Antibodies against *R. salmoninarum* were detected in sera of Chinook salmon, coho salmon, and steelhead returning to spawn at LMRW and PRW (Figure 4.6). These antibodies were detected in the majority of the Chinook salmon and steelhead, and to a lesser degree, in the coho salmon (Figure 4.6; Table 4.1). Overall, the distribution of antibody levels for most of the fish groups appeared to be positively skewed, with the larger proportion of anti-*R. salmoninarum* antibody-producing fish occurring in the upper quartile (Figure 4.6). Overall, Chinook salmon produced a wide range of consistently similar levels of antibodies in both 2009 and 2013, which tended to be greater than the median antibody level (Figure 4.6). However, in 2009, the mean antibody response of female Chinook salmon was significantly greater than the male antibody response ($F = 13.01$; $df = 1, 58$; $P = 0.006$). Also, a larger range of antibody levels for male steelhead was observed in 2013 compared to 2009, with the most variation occurring in the upper quartile (Figure 4.6). Furthermore, the mean antibody response of male steelhead was significantly greater than female steelhead in 2013 ($F = 14.93$; $df = 1, 58$; $P = 0.0002$). In

2009 and 2013, considerably less variation was observed in the levels of antibodies detected in male coho salmon, with the exception of one individual in 2009 with an OD value of 1.568 (Figure 4.6). Alternatively, a substantially wider range of antibody levels was observed in female coho salmon from 2013 than 2009, with the majority of antibody-producing fish occurring in the upper quartile (Figure 4.6).

Interestingly, the *R. salmoninarum* p57 antigen was only detected in two of the kidneys and spleens from Chinook salmon that were producing antibodies, at a medium and high intensity (Table 4.1). Similarly, one steelhead had a medium intensity infection in the kidneys and spleen and was also producing antibodies (Table 4.1). However, while p57 was not detected in the kidneys and spleen of any coho salmon, it was detected at medium and low intensities in the reproductive fluids of one male and three female fish (Table 4.1). Interestingly, none of the gamete-infected individuals were producing detectable levels of anti-*R. salmoninarum* antibodies (Table 4.1).

4.4. Potential protective role of circulating binding antibodies to challenge with live *R. salmoninarum*

Antibodies were detected in the lethargic and dying fish in both NPn and NPi groups with the exception of week 30 p.i. (Table 4.2). Antibody levels in the NPn group reached up to 0.270 OD value, while antibodies detected in the NPi fish group showed a much higher response, with OD values as high as 0.608 (Table 4.2). The antibody response in both fish was the highest at weeks 7 and 8 p.i. (Table 4.2), which was also when the mortalities diminished

(Figure 5.7). Similar to the NF and SF groups of fish, the *R. salmoninarum* p57 antigen was detected more frequently earlier in the challenge as well and was not detected at all after week 7 p.i. (Table 4.2).

The Kaplan Meier analysis revealed that the difference in the overall survivability of the NPn fish group was not significantly different from the NPi group (χ^2 = 0.0524, df = 1, *P* = 0.8189). The mean time-to-death was longer for the NPi fish [22.6 weeks (SE ± 2.5019)] compared to the NPn fish [6.1 weeks (SE ± 0.2939)]; however, due to the small number of mortalities that occurred, this was not deemed significant. Also, over 60% of fish from both groups infected with *R. salmoninarum* survived until week 33 p.i. when the challenge was terminated (Table 4.2).

5. Discussion

The indirect ELISA protocol used in this study consistently detected circulating anti-*R. salmoninarum* antibodies in the majority of tested fish sera. The modified protocol was based upon the two earlier studies of Wood and Kaattari (1996) and Alcorn and Pascho (2000). While the modified protocol utilized the same coating and blocking strategies developed by Wood and Kaattari (1996), a single dilution of each test serum (s recommended by Alcorn and Pascho (2000) was used in this protocol, as opposed to five dilutions of each test serum per assay used by Wood and Kaattari (1996). This modification enabled the testing of 40 samples on one ELISA microplate as opposed to ~8 samples/plate (Wood and Kaattari, 1996), without compromising the sensitivity and specificity of the assay. As such, the modified protocol reduces costs of reagents, potentially reduces the error inherent to serial dilutions, and is relatively

uncomplicated to execute. Also, the Wood and Kaattari (1996) protocol used the biotinylated mouse anti-trout immunoglobulin monoclonal antibody 1-14, while the modified protocol uses a commercially available monoclonal antibody that is directed against the heavy chain of *Oncorhynchus* spp., a matter that allowed testing of rainbow trout/steelhead trout, Chinook salmon, and coho salmon. Further, the use of a tertiary alkaline phosphatase-conjugated goat-anti-mouse antibody allowed more amplification and consequently better visualization of the reaction. Lastly, microplates from the modified ELISA were read a single time at 405 nm, compared to the kinetic read performed by Wood and Kaattari (1996) at the same wavelength, thereby saving 10 minutes per microplate.

In this study, four experimentally infected groups were arbitrarily used to generate fish specific anti-*R. salmoninarum* circulating antibodies that could be used to test the modified protocol. The antibodies were detected in the four fish groups as early as week 3 p.i. and lasted until the 26th week p.i., when the observation period ended. However, in most of the fish groups, elevated levels of antibody production were short-lived and elevated long-term production of this particular fish antibody type did not appear to occur. Also, the maximum antibody responses were associated with either an ongoing or recovering *R. salmoninarum* infection, with declining production of antibodies within 15-18 weeks p.i. Without extending the study period, it is unknown how long antibody production would persist, but it most likely would not have continued. Alcorn et al. (2005) observed a decline in the detection of anti-*R. salmoninarum* antibodies in experimentally infected Atlantic salmon after 11 weeks post infection, as did Jansson and Ljungberg (1998) after 8 weeks post-infection. To this end, the production of antibodies observed in experimental fish from this study is likely a transient

response to an infection with live *R. salmoninarum*. The extent to which protection is conferred by this antibody type appears to be minimal.

Unexpectedly, antibody levels in spawning fish were considerably higher than in experimental fish. Indeed, there are a handful studies that have attempted to assess the presence of antibodies to *R. salmoninarum* in adult feral or wild salmonids. Bartholomew et al. (1991) detected anti-*R. salmoninarum* antibodies in naturally infected spring Chinook salmon and coho salmon using Western blot methods, describing the levels as low titers. Additionally, a weak antibody response against *R. salmoninarum* was observed in adult Atlantic salmon and rainbow trout from fish farms with a history of clinical BKD (Jansson and Ljungberg, 1998). The authors hypothesized that the weak antibody response could have been due to lower water temperatures throughout their study (8°C) in the farms.

The assay modified in this study detected antibodies against *R. salmoninarum* in feral Chinook salmon, coho salmon, and steelhead that were returning to spawn at Lake Michigan weirs in 2009 and 2013 with relatively high levels. The extremely low presence of the p57 antigen coincides with the observed decline of BKD in the GLB (Schulz, Chapter 2). Despite the lack of detection of p57 in the kidneys, ~80% of the feral fish were producing detectable antibodies against *R. salmoninarum* at higher levels than the experimentally challenged fish. It is possible that the feral Chinook salmon, coho salmon, and steelhead are being exposed to *R. salmoninarum* while residing in the Great Lakes and are becoming re-infected with the pathogen due to its presence in other fish species (Faisal et al., 2010b). This data suggests that the spawning-stressed fish are prevailing against this serious pathogen as they find their way to their spawning grounds. Throughout this study, the ELISA-whole bacterium binding antibodies

were the only host defense mechanism measured. Fish are known to combat *R. salmoninarum* through multiple immune molecules and phagocytic cells (Young and Chapman, 1978; Secombes, 1985; Hardie et al., 1996; Grayson et al., 2002), which can be more potent and long lasting compared to the antibodies measured in this study.

APPENDIX

Table 4.1. Summary of the presence of anti-*Renibacterium salmoninarum* antibodies, p57 antigen, and the bacterium in blood and kidney and spleen samples from experimentally and naturally infected *Oncorhynchus* spp. An indirect enzyme-linked immunosorbent assay (ELISA) was used to determine the number of fish producing antibodies (No. Ab+ fish) with the resultant mean optical density (OD) value. Active infections with *R. salmoninarum* were assessed by bacterial culture [No. K/S+ fish culture)] and sandwich ELISA [No. K/S+ fish (ELISA)]. The intensity of infection as determined by ELISA was designated as low (L), medium (M), or high (H). ND = not determined.

Fish species	No. fish sampled	No. Ab+ fish	Mean OD value	No. K/S+ fish (ELISA)	No. K/S+ fish (culture)
Rainbow trout					
Naïve (NFn)	86	43/86	0.351	18/86 (4H-7M-7L)	17/86
Naïve, bacterin (NFi)	85	44/85	0.234	23/85 (2H-12M-9L)	16/85
Survivor (SFn)	85	41/82	0.188	17/85 (3H-11M-3L)	16/85
Survivor, bacterin (SFi)	84	55/84	0.298	18/86 (5H-8M-5L)	23/84
Naïve (NPn)	22	10/22	0.125	7/22 (2H-5M)	7/22
Naïve, bacterin (NPi)	23	14/24	0.198	4/24 (2H-2M)	10/23
Chinook salmon					
Male	30	30/30	0.607	1/30 (M)	ND
Female	30	30/30	0.660	1/30 (H)	ND
Coho salmon					
Male	20	7/20	0.203	0/20	ND
Female	20	8/20	0.266	0/20	ND
Steelhead					
Male	30	28/30	0.394	1/30 (1M)	ND
Female	30	26/30	0.297	0/30	ND

Table 4.2. Summary of the survival of naïve non-immunized (NPn) and naïve immunized (NPi) rainbow trout (*Oncorhynchus mykiss*) in the weeks post-infection with *Renibacterium salmoninarum*. An indirect enzyme-linked immunosorbent assay (ELISA) was used to determine the number of fish producing antibodies (No. Ab+ fish) with the resultant mean optical density (OD) value. Active infections with *R. salmoninarum* were assessed by bacterial culture [No. K/S+ fish culture)] and sandwich ELISA [No. K/S+ fish (ELISA)]. The intensity of infection as determined by ELISA was designated as low (L), medium (M), or high (H).

Weeks post-infection	Group	No. of mortalities	No. Ab+ fish	Mean OD value	No. K/S+ fish (ELISA)	No. K/S+ fish (culture)
4	NPn	5	1/5	0.076	5 (3M-2H)	1/1 fish
5	NPn	2	1/2	0.102	2 (2M)	2/2 fish
7	NPn	1	1/1	0.270	0	1/1 fish
33*	NPn	14	7/14	0.135	0	3/14 fish
3	NPi	1	1/1	0.105	1 (H)	1/1 fish
4	NPi	2	1/2	0.080	2 (1M-1H)	2/2 fish
6	NPi	2	1/2	0.158	0	2/2 fish
7	NPi	1	1/1	0.604	1 (1M)	1/2 fish
8	NPi	2	1/2	0.608	0	1/2 fish
30	NPi	1	0/1	0.061	0	1/1 fish
33*	NPi	15	9/15	0.151	0	2/15 fish

*All remaining fish were euthanized on Week 33 and the challenge was terminated.

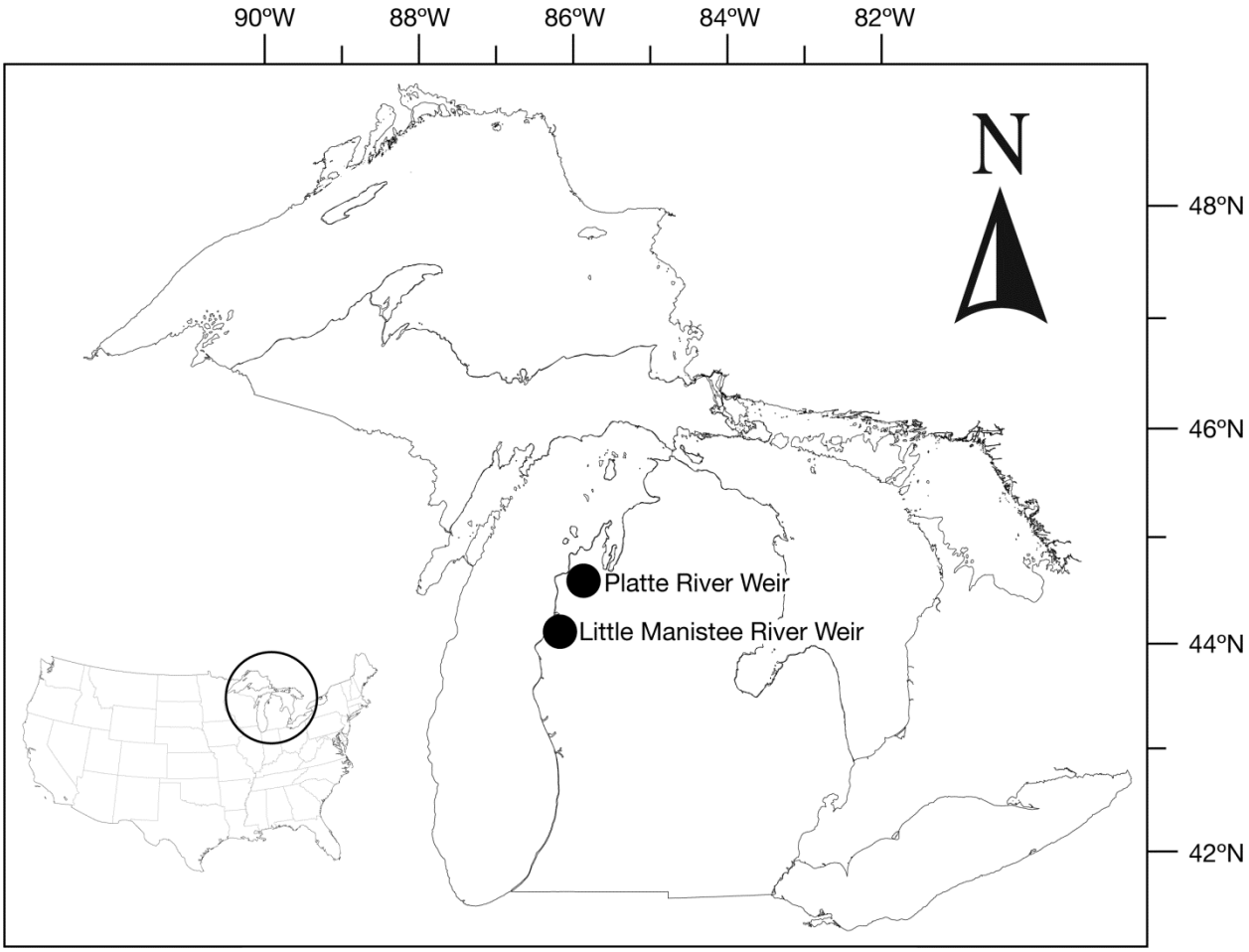

Figure 4.1. The Michigan Department of Natural Resources gamete-collecting weirs where Chinook (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), and steelhead (*O. mykiss*) were collected in 2009 and 2013: the Little Manistee River Weir (Chinook salmon and steelhead) and the Platte River Weir (coho salmon).

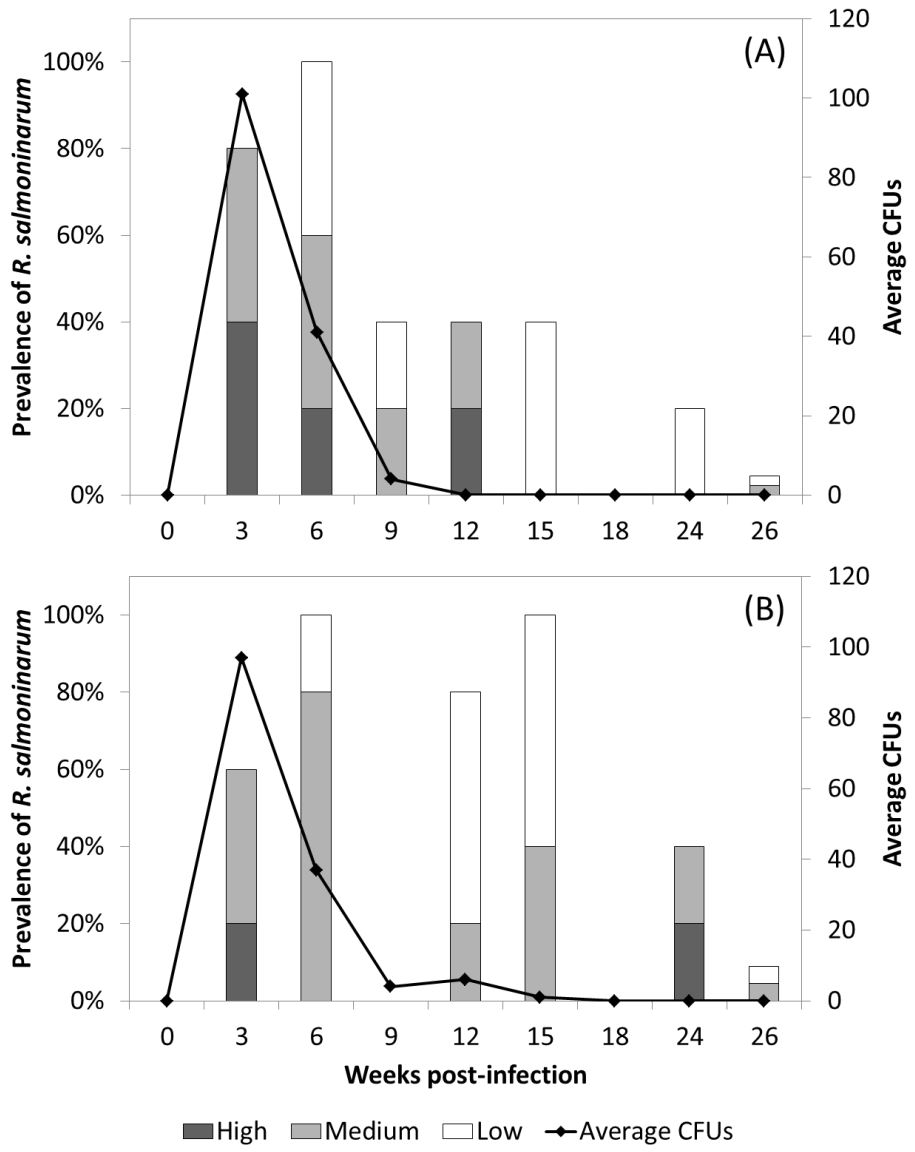

Figure 4.2. The prevalence of *Renibacterium salmoninarum* in the kidney/spleen tissue samples of A) 8 month old naïve rainbow trout (*Oncorhynchus mykiss*), and B) age cohort rainbow trout that have received two doses of *R. salmoninarum* bacterin *per os* at each sampling period (weeks post-infection with live bacteria). *Renibacterium salmoninarum* and its p57 antigen were detected by the sandwich enzyme-linked immunosorbent assay, with the infection intensity expressed as the proportion of fish exhibiting low, medium, and high levels of infection (n=5 per sampling periods; n=45 at week 26 p.i.). The average number of *R. salmoninarum* colony forming units (CFUs) isolated from kidney is depicted as a line graph.

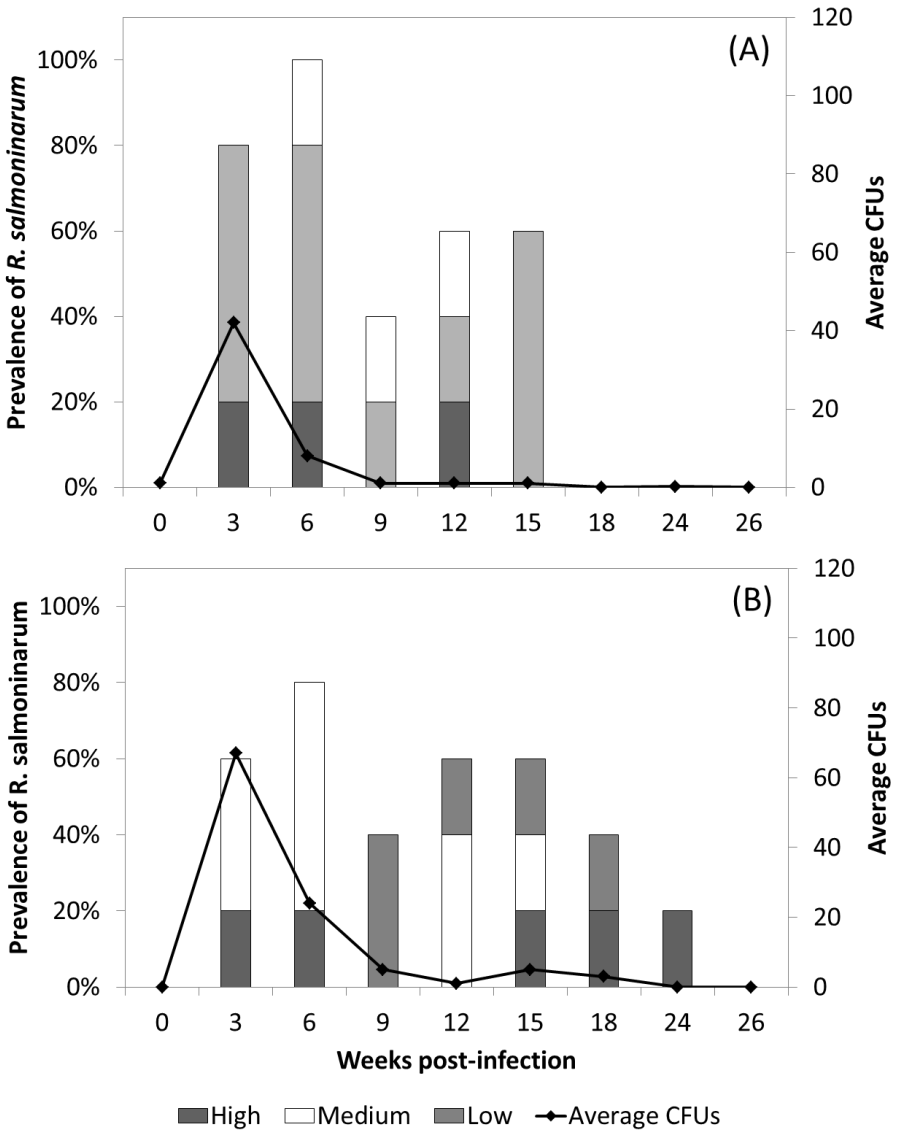

Figure 4.3. The prevalence of *Renibacterium salmoninarum* in the kidney/spleen tissue samples of A) 11 month old rainbow trout that survived an infection with live *R. salmoninarum* 16 weeks prior to being used in this experiment B) age cohort survived rainbow trout that have received two doses of *R. salmoninarum* bacterin *per os* at each sampling period (weeks post-infection with live bacteria). *Renibacterium salmoninarum* and its p57 antigen were detected by the sandwich enzyme-linked immunosorbent assay, with the infection intensity expressed as the proportion of fish exhibiting low, medium, and high levels of infection (n=5 per sampling period; n=44-45 at week 26 p.i.). The average number of *R. salmoninarum* colony forming units (CFUs) isolated from kidney is depicted as a line graph.

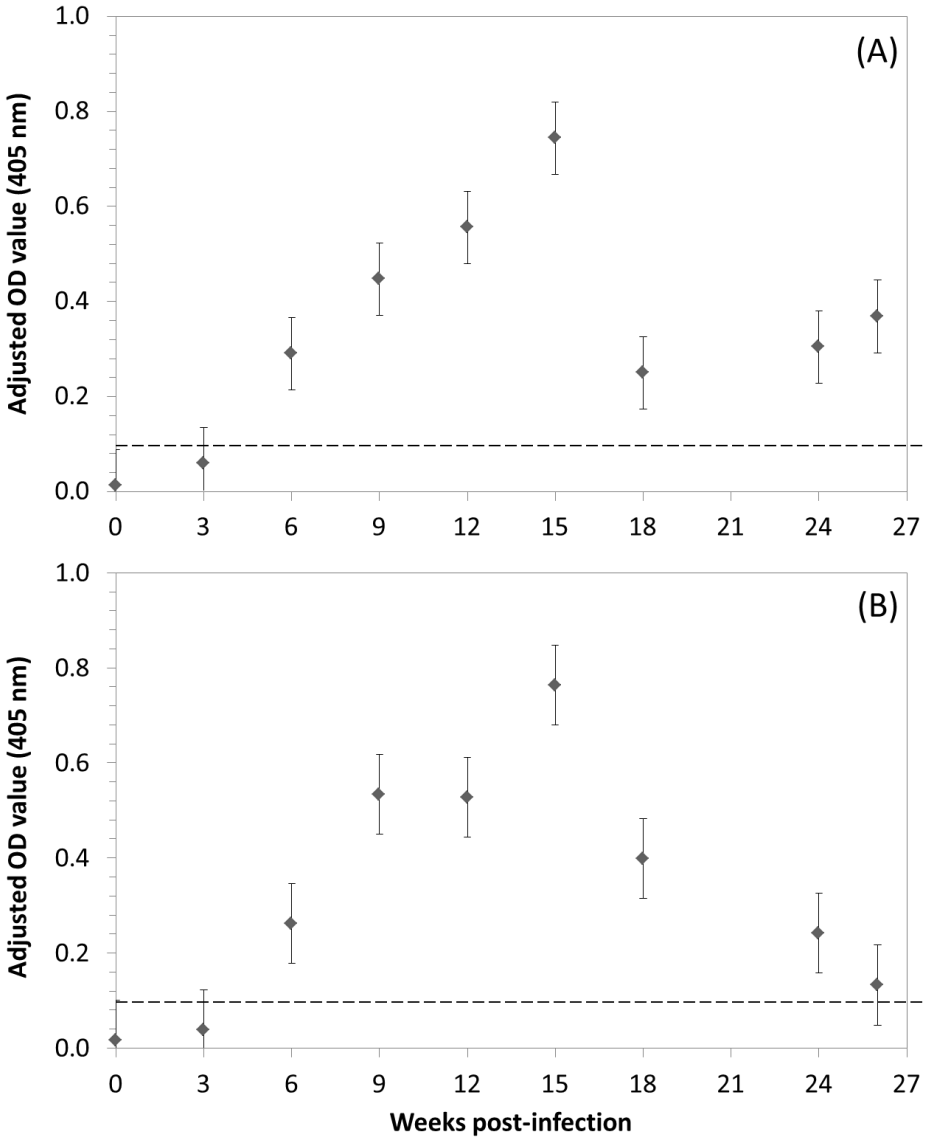

Figure 4.4 The mean antibody response (± SE) of A) 8 month old naïve rainbow trout (*Oncorhynchus mykiss*), and B) age cohort rainbow trout that have received two doses of a *R. salmoninarum* bacterin *per os* at each sampling period (weeks post-infection with live bacteria). The average optical density (OD) value was used to evaluate the production of anti-*R. salmoninarum* antibodies in fish. A separate group of naïve fish were used to determine the positive-negative threshold (dashed line), which was the average OD value plus two standard deviations (i.e., 0.094).

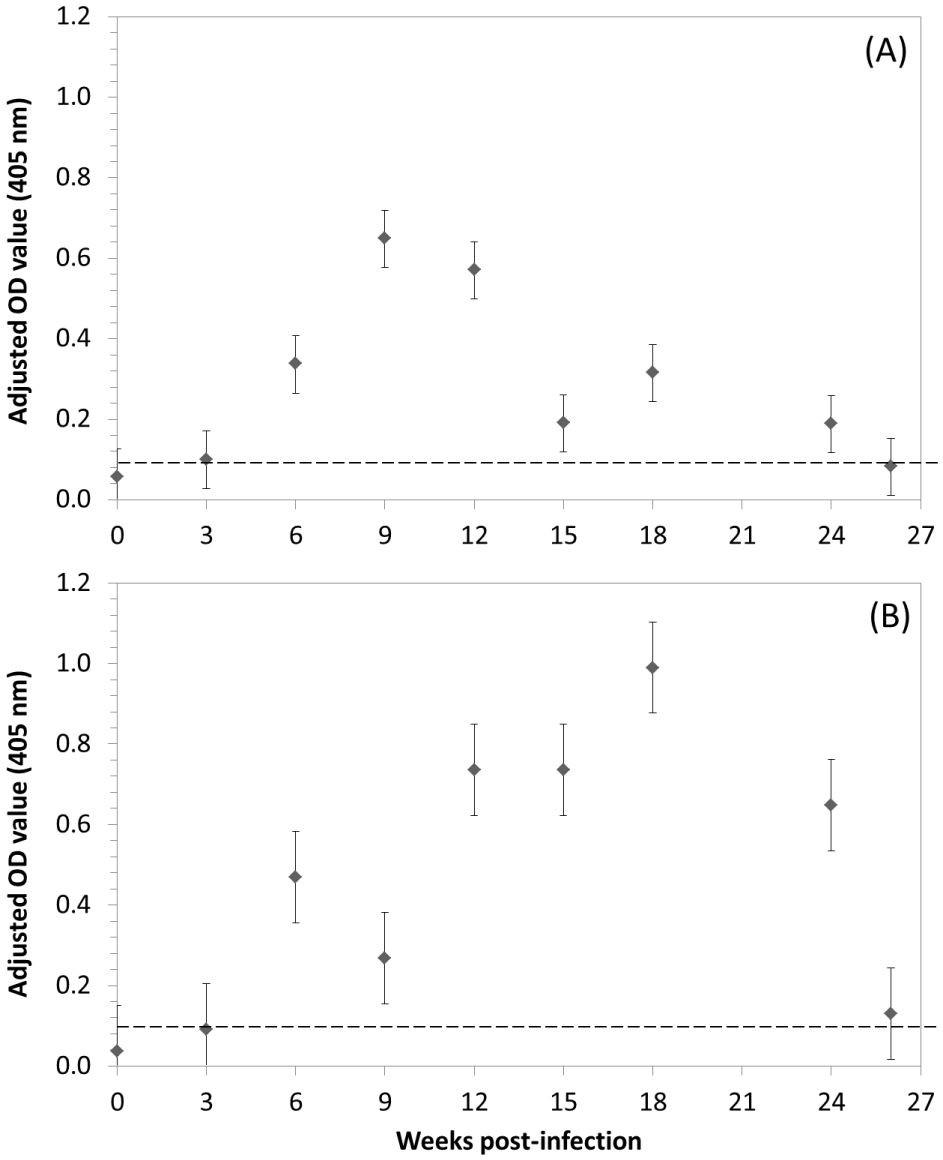

Figure 4.5. The mean antibody response (± SE) of A) 11 month old rainbow trout that survived an infection with live *R. salmoninarum* 16 weeks prior to being used in this experiment B) age cohort survived rainbow trout that have received two doses of a *R. salmoninarum* bacterin *per os* at each sampling period (weeks post-infection with live bacteria). The average optical density (OD) value was used to evaluate the production of anti-*R. salmoninarum* antibodies in fish. A separate group of naïve fish were used to determine the positive-negative threshold, which was the average OD value plus two standard deviations (i.e., 0.094).

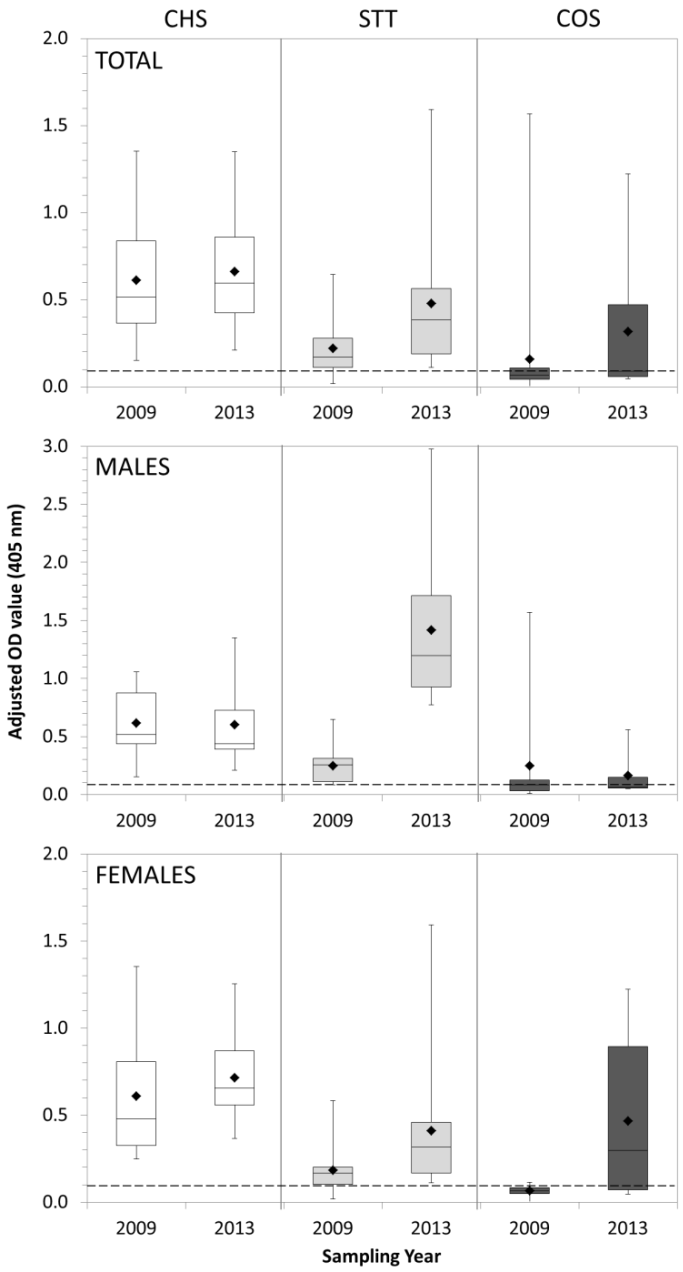

Figure 4.6. The mean circulating antibody levels of Chinook salmon (*Oncorhynchus tshawytscha*), steelhead (*O. mykiss*), and coho salmon (*O. kisutch*) returning to spawn at the Little Manistee River Weir and the Platte River Weir. The data are presented as box and whisker plots, where the central box contains the interquartile range and the median is represented as a horizontal bar, which divides the interquartile range. The maximum 'whisker' length is the maximum OD value and the minimum 'whisker' length is the minimum OD value. The average optical density (OD) value was used to evaluate the production of anti-*R. salmoninarum* antibodies in fish. A separate group of naïve fish were used to determine the positive-negative threshold (dashed line), which was the average OD value plus two standard deviations (i.e., 0.094).

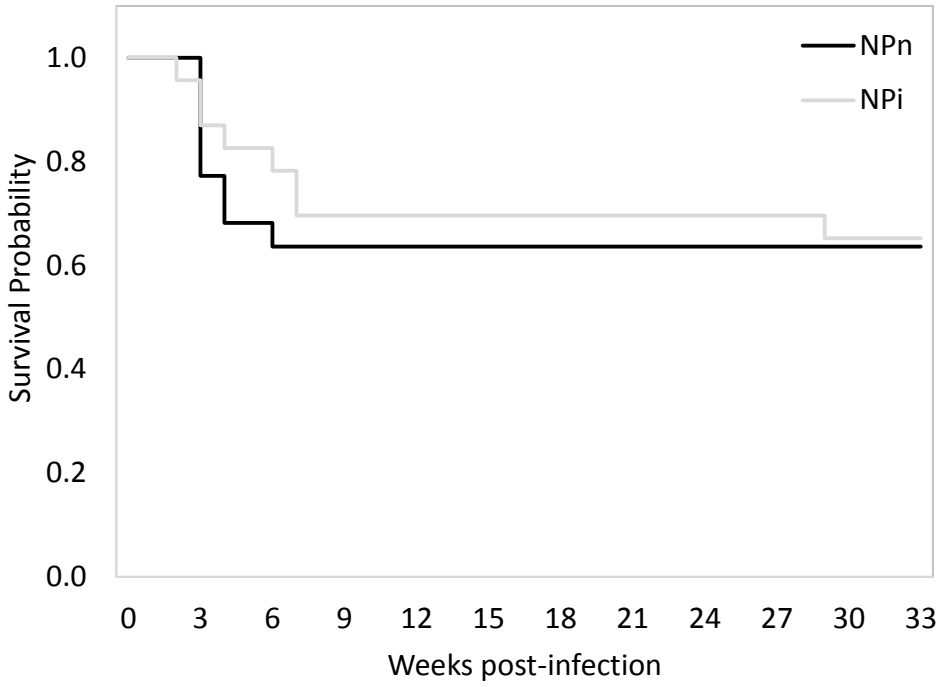

Figure 4.7. The survival probability of 11 month old naïve rainbow trout (*Oncorhynchus mykiss*) (NPn) and age cohort rainbow trout that have received one dose of *R. salmoninarum* bacterin *per os* (NPi) in the weeks post-infection with live *Renibacterium salmoninarum*.

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Chapter 5

Efficacy of Current Testing Procedures of Spawning Chinook Salmon (*Oncorhynchus tshawytscha*) in Minimizing the Introduction of *Renibacterium salmoninarum* into Michigan Hatcheries

1. Abstract

Bacterial kidney disease (BKD), caused by *Renibacterium salmoninarum*, has been associated with wide-scale mortalities in Chinook salmon (*Oncorhynchus mykiss*) from the Great Lakes basin, which resulted in the adoption of stricter biosecurity practices at Michigan Department of Natural Resources (MDNR) gamete-collecting weirs. The purpose of this study was to determine prevalence and intensity of *R. salmoninarum* in male and female Chinook salmon returning to spawn at four gamete-collecting weirs in Michigan: the Boardman River Weir, Little Manistee River Weir (LMRW), and Medusa Creek Weir (Lake Michigan watershed), and the Swan River Weir (Lake Huron watershed). Also, the extent to which infected fish could be shedding the bacterium within the gametes was determined, as well as if infected fish were exhibiting different diagnostic patterns indicative of a progressing *R. salmoninarum* infection. Nested polymerase chain reaction (nPCR), quantitative enzyme-linked immunosorbent assay (Q-ELISA), and bacterial culture were used to assess prevalence of *R. salmoninarum* in a kidney and spleen homogenate and the gametes, while the presence of circulating antibodies was determined by agglutination. Among the four weirs, SRW had the highest prevalence of *R. salmoninarum*, with female Chinook salmon appearing to be more susceptible to BKD infection than male Chinook salmon. Prevalence of *R. salmoninarum* in the gametes from both sexes was comparatively low, but there was evidence that males may contribute to shedding. Using the results from nPCR, Q-ELISA, culture, and agglutination, individuals were placed into one of six disease stages. Evidence of disease progression was observed at LMRW, with earlier disease stages and more intense infections occurring later in the spawning run compared to earlier. It

is suggested that managers consider harvesting gametes used for propagation during the early spawning run, to reduce the influx of *R. salmoninarum* being introduced into hatcheries.

2. Introduction

Renibacterium salmoninarum, the causative agent of bacterial kidney disease (BKD) in salmonids, is an obligate intracellular pathogen that is transmitted both horizontally (Mitchum and Sherman, 1981; Bell et al., 1984) and vertically (Evelyn et al., 1984, 1986). In the 1980s, *R. salmoninarum* was associated with wide-scale mortalities in Chinook salmon (*Oncorhynchus mykiss*) from Lake Michigan (Holey et al., 1998). These unexpected BKD-epizootics highlighted the lack of knowledge on the host defense mechanisms of Chinook salmon, particularly in its new habitat in the Great Lakes basin. The majority of knowledge regarding *R. salmoninarum* and Chinook salmon interactions has originated from studies performed in the Pacific Northwest, where the fish alternate between marine and freshwater environments. However, in the Great Lakes basin, there is no access to a marine environment, thus resulting in different physiological conditions for Chinook salmon.

To control BKD outbreaks and minimize the spread of *R. salmoninarum* in the Great Lakes basin, natural resources managers adopted a stringent procedure including a visual inspection of each spawning female and the subsequent culling of any gametes previously collected from spawning females with overt signs of clinical BKD. In addition, the kidneys of individual fish were routinely examined for the presence of *R. salmoninarum* soluble antigens using a monoclonal antibody based Field ELISA (FELISA). This rapid field test allowed the

detection of fish whose kidneys were laden with *R. salmoninarum* antigen, resulting in the exclusion of their eggs from further incubation and propagation (Beyerle and Hnath, 2002; Faisal and Hnath, 2005). The health inspection and the FELISA were not performed on males due to the published studies of Klontz (1983) and Evelyn et al. (1986), which have minimized the role of male fish in vertical transmission of *R. salmoninarum*. However, more recently Eissa et al. (2007) demonstrated similar levels of *R. salmoninarum* infection in milt and ovarian fluid from naturally infected brook trout and suggested that males could contribute to vertical transmission of BKD. Moreover, at certain gamete-collecting weirs in Michigan, Schulz (Chapter 2) reported a higher incidence of *R. salmoninarum* infection in milt than ovarian fluid from naturally infected salmon, implying that males could be contributing to the vertical transmission of *R. salmoninarum*, although to what extent this is occurring is not known.

Over the last three decades, a number of diagnostic assays have been developed to determine the presence of *R. salmoninarum* or its soluble antigens in infected fish tissues, such as culture on selective media, quantitative ELISA (Q-ELISA), and nested polymerase chain reaction (nPCR) (Pascho and Elliott, 2004). However, when multiple diagnostic tests are performed on the same sample, it is not uncommon for numerous discrepancies among findings to occur (Cipriano et al., 1985; Sakai et al., 1989; White et al., 1995; Jansson et al., 1996; Miriam et al., 1997; Pascho et al., 1998). This issue can often make findings, and the true infectious status of a fish, difficult to interpret. However, considering that each assay (culture, Q-ELISA, and nPCR) detects a different characteristic of the pathogen, the conflicting results could be related to the viability of *R. salmoninarum*. Recently, Faisal and Eissa (2009) analyzed the discrepancies among diagnostic assays in four salmonid stocks and suggested that the

observed inconsistencies could be due to the phase of an *R. salmoninarum* infection at the time of sampling. For example, as nPCR detects the presence of bacterial DNA, a fish that was positive via nPCR only was suggested to be in an early infection (Faisal and Eissa, 2009). Conversely, Q-ELISA detects antigen released from the bacterium, thereby not requiring an active infection for detection; therefore, a fish that was positive by Q-ELISA only reflected a late, or recovering, infection (Faisal and Eissa, 2009). Not only does this further the understanding of the pathogenicity of *R. salmoninarum*, but being aware of the current stage of infection occurring in a fish population could influence the control measures implemented by hatchery managers.

The present study has been designed in order to better understand the epizootiology of *R. salmoninarum* infection in Michigan's Chinook salmon returning to spawn in the gamete collecting facilities and to determine how effective the current *R. salmoninarum* testing and culling program is in minimizing the transmission of *R. salmoninarum*. Specifically, the objectives are 1) to determine the prevalence and intensity of *R. salmoninarum* in spawning Chinook salmon stocks returning to Michigan's weirs; 2) to determine the extent to which infected fish shed the bacterium with the gametes; and 3) to determine if infected fish are exhibiting different diagnostic patterns indicative of progressive *R. salmoninarum* infection. This novel approach will allow the elucidation of important aspects in the dynamics of *R. salmoninarum* infections in Chinook salmon, one of the most important fish species in the Great Lakes basin.

3. Material and Methods

3.1. Fish collection

During September and October of 2005, returning Chinook salmon spawners were collected from four gamete collecting weirs in Michigan: the Little Manistee River Weir (LMRW), the Medusa Creek Weir (MCW), and the Boardman River Weir (BRW), are all within the Lake Michigan watershed, while the Swan River Weir (SRW) resided within the Lake Huron watershed (Figure 5.1). Fish were collected from the LMRW on five separate occasions; September 20 (30 males, 30 females), October 4 (56 males, 30 females), October 5 (57 males, 22 females), October 6 (60 males, 44 females), and October 25 (55 males, 42 females). Fish were collected on October 14 from the BRW (30 males, 30 females) and the MCW (30 males, 30 females), while the MCW was also sampled on October 20 (36 males, 18 females). The SRW was sampled once on October 3 (30 males, 30 females).

At the time of collection, the male Chinook salmon ranged in weight from 0.6 to 20 kg, with an average weight of 7.2 kg (± 3.8 kg) and ranged in length from 42 cm to 102.6 cm, with an average length of 75.2 cm (± 10.9 cm). Also, female Chinook salmon ranged in weight from 1.4 to 14.3 kg with an average weight of 6.4 kg (± 2.5 kg) and ranged in length from 53 to 92 cm, with an average length of 77.3 cm (± 7.0 cm).

3.2. Sample collection

Prior to sample collection, Chinook salmon were euthanized by immersion in CO_2-laden water and manual blunt force trauma to the cranium. Gametes were then harvested by Michigan Department of Natural Resources (MDNR) personnel for hatchery propagation, with a sub-sample collected in sterile 15 ml centrifuge tubes (Denville Scientific Inc., Metuchen, NJ) for detection of *R. salmoninarum* with Q-ELISA (described below). Gamete samples were stored at -20°C until processing. Blood was collected from the dorsal aorta at the caudal peduncle region and allowed to clot in sterile tubes. Length, weight, and sex of all fish were recorded and external examinations were performed. Fish were externally disinfected with 70% ethanol and subjected to thorough internal clinical examinations and aseptic target tissue collection. Sterile individual forceps and scissors were used for each fish tissue collection.

In addition to the gametes, samples (< 5 g) of kidneys and spleens were removed in the field and stored on ice in whirlpaks (VWR International, West Chester, PA), as recommended by the American Fisheries Society-Fish Health Section Bluebook (2012) and the World Organization for Animal Health (2012), and frozen at -20°C until processing. Kidney and spleen homogenates were tested for the presence of *R. salmoninarum* by culture, nPCR, and Q-ELISA.

3.3. Bacterial culture and isolation

Renibacterium salmoninarum isolation was performed by streaking 1-10 µl of the target tissue onto modified kidney disease medium (MKDM; Faisal et al., 2010) agar plates using

sterile disposable inoculating loops and incubating the plates under aerobic conditions at 15°C for a total of 42 days. The kidney/spleen samples were diluted 1:10 (weight/volume) in sterile phosphate buffered saline (PBS; pH 7.2) and suspended by repeated expulsion through a sterile pipette. The suspension was then diluted via 10- fold serial dilutions and 10 µl of each dilution (e.g., undiluted to 10^{-8}) was dispensed onto the MKDM plates. Plates were incubated at 15°C and were observed for typical *R. salmoninarum* growth every 7 days for a total of 6 weeks. Colonies were observed under a dissecting microscope for typical *R. salmoninarum* morphological characteristics: convex, cream-colored, round, and smooth (Austin and Austin, 2007). Colonies that fit this criterion were assessed by additional biochemical testing, which included Gram stain, cytochrome oxidase, and catalase reaction, which are key biochemical tests used to identify *R. salmoninarum* (Sanders and Fryer, 1980). Colonies that were Gram positive, cytochrome oxidase negative, and catalase positive were then confirmed molecularly via nPCR (see below).

3.4. Extraction of DNA

The DNA extracted from the kidney and spleen samples was processed according to the DNeasy® Blood and Tissue Kit (Qiagen) instructions for animal tissue. Also, as recommended by the kit instructions, the pre-treatment for Gram-positive bacteria (including the lysis buffer) was applied to all of the samples. After extraction, the DNA was then quantified with the Qubit® Fluorometer (Life Technologies, Grand Island, NY) and diluted to a 20 ng/µl concentration.

3.5. Nested PCR

The nPCR method and primers recommended by Pascho et al. (1998) were used initially, with minor modifications. To optimize the protocol to the laboratory's conditions, the annealing temperature was changed to 60°C for DNA extracted from pure bacterial cultures and kidney/spleen tissues (based upon optimization experiments). Additionally, the total reaction volume was reduced from 50 µl to 25 µl, consisting of 1 µl each of template DNA (20 ng total), forward primer (10 µmol), and reverse primer (10 µmol), as well as 12.5 µl of GoTaq® Green Master Mix (Promega Corp., Madison, WI) and 9.5 µl of nuclease-free water. The controls were composed of a PCR mixture containing water instead of DNA template (negative control) and DNA from a pure culture of *R. salmoninarum* ATCC #33209 strain (positive control). Five microliters of the nPCR products and controls were mixed with 1 µl of SYBR Green II RNA Gel Stain (Cambrex Bio Science Rockland, Inc., East Rutherford, NJ) and loaded into a gel consisting of 2% Ultra Pure™ agarose (Invitrogen, Grand Island, NY). Each electrophoresis gel included 5 µl of a 1 Kb plus ladder (Invitrogen) mixed with 1 µl of 6X gel loading dye (New England Bio Labs, Inc., Ipswich, MA). Gels were run at 100 v for 35 minutes in 1X Tris-Acetate Buffer (Sigma-Aldrich Corp., St. Louis, MO) in a Gel XL Ultra V-2 electrophoresis box (Labnet) and a Sub-Cell GT electrophoresis box (Bio Rad Laboratories, Inc., Hercules, CA). Gels were visualized with a Canon G10 camera and UV Trans-Illuminator. Samples were considered positive for *R. salmoninarum* when a 320-bp band was present (Pascho et al., 1998).

3.6. Q-ELISA

The general Q-ELISA protocol outlined in Pascho and Mulcahy (1987), with modifications recommended by Gudmundsdóttir et al. (1993) and Olea et al. (1993), was used to assess *R. salmoninarum* antigens in the kidney/spleen samples. Prior to the Q-ELISA procedure, kidney/spleen tissue samples from the immersion and injection challenge were diluted 1:8 (w:v) with Hank's Balanced Salt Solution (HBSS; Sigma), while kidney/spleen samples from naturally infected fish were diluted 1:4 with HBSS. The samples were then stomached on high speed for two minutes with the Biomaster Stomacher (Wolf Laboratories Limited, Pocklington, York, UK). An aliquot of 250 µl of stomached kidney/spleen samples were dispensed into 1.5ml microcentrifuge tubes containing 250 µl of phosphate buffered saline with Tween-20 (PBS-T20; Sigma) and 5% goat serum (Sigma) and 50 µl of CitriSolv (Fisher Scientific, Pittsburgh, PA). The purpose of the CitriSolv solvent was to dissolve and remove liquids form the aqueous supernatant (Gudmundsdóttir et al., 1993), while the introduction of 5% goat serum was to increase sensitivity of the assay (Olea et al., 1993). Samples were vortexed for approximately 10 sec, heated at 100°C for 15 min, and then centrifuged at 14,000 rpm for 10 min. The aqueous supernatant of each sample was used for Q-ELISA testing.

The positive-negative cutoff absorbance for the samples was 0.10. Samples that tested positive were assigned the following antigen levels: low (0.10-0.199), medium (0.20-0.999), and high (≥1.00), as recommended by Meyers et al. (1993) and Pascho et al. (1998). Each assay included two negative controls, a negative fish tissue sample and a dilution buffer, and two

positive controls, a positive fish tissue sample and a *R. salmoninarum* positive control (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD).

3.7. Agglutination assay

The agglutination assay to detect and quantify Chinook salmon circulating antibodies against *R. salmoninarum* was performed according to the protocol originally described by Evelyn (1971), with few modifications. The antigen was prepared from cryo-preserved *R. salmoninarum* (ATCC #33209) that was revived on MKDM. Following 5-7 days of incubation at 15°C, bacterial growth was centrifuged at 3000 rpm for 10 min, washed three times with sterile 0.9% NaCl saline. Washed bacteria were then re-suspended in saline at a concentration of 50 mg ml^{-1}. Bacteria were then killed by heating the suspension to 62°C for 45 min in a water-bath.

Standard two-fold serial dilutions of serum were made using sterile saline as the diluent. The agglutinin test was performed in tubes with the heat-killed *R. salmoninarum* as the antigen and was adjusted to a concentration yielding an optical density of 1.25 at 420 mμ. A positive reaction was indicated by the formation of macroscopic clumping of the antigen following incubation with serum samples overnight at 4°C. A titer (expressed as the reciprocal of the highest serum dilution showing positive reaction) was determined for each tested serum sample. Sera showing no agglutination at a dilution of 1:2 were considered negative.

3.8. Statistical analyses

To assess potential shedding, individuals were separated into one of four infection categories based upon Q-ELISA results from the kidney and spleen homogenate and gamete sample: (1) individuals that were negative for *R. salmoninarum* in the kidney and spleen homogenate and the gamete sample (ks-/gam-); (2) individuals that were positive for *R. salmoninarum* in the kidney and spleen homogenate and negative in the gamete sample (ks+/g-); (3) individuals that were negative for *R. salmoninarum* in the kidney and spleen homogenate and positive in the gamete sample (ks-/gam+); and (4) individuals that were positive for *R. salmoninarum* in the kidney and spleen homogenate and the gamete sample (ks+/gam+). Individuals that were positive for *R. salmoninarum* in the gamete sample were considered to be potential shedders of the bacteria.

To explore potential disease progression, samples were placed into one of six disease stages (Table 5.1) depending on the culture, Q-ELISA, nPCR, and agglutination results, based on the study of Faisal and Eissa (2009). Disease stage scores (1-6) were analyzed using a general linear model (GLM) based on a cumulative logistic link function. The GLM included the effects of period and gender and their interaction, as well as weight; other covariates such as length and condition factor were investigated but they were not statistically significant due to the high collinearity with weight. Estimated differences between different groups were reported as odds ratios of being in lower numbered categories. SAS PROC GLIMMIX and SAS statistical software (SAS Institute, Inc., Cary, NC) was used for data analysis. Analyses of probabilities of co-infection in kidney and gametes and associations between the various disease stage

categories and the presence or absence of *R. salmoninarum* in the gametes were further investigated using contingency chi-square tests or Fisher's exact tests when sample sizes in disease stage categories were small.

4. Results

4.1. Prevalence and semi-quantitation of *R. salmoninarum* by Q-ELISA

The Q-ELISA was performed on CHS returning to all four weirs (BRW, LMRW, MCW, and SRW). The prevalence of *R. salmoninarum* detected from the combined four weirs was 6.5% (43/660 fish). While not statistically significant ($F = 1.71$; df = 6, 654; $P = 0.1161$), SRW exhibited highest prevalence of *R. salmoninarum* at 10%, followed by MCW at 7%, BRW at 6.7%, and LMRW at 5.9% (Figure 5.2A). The highest intensity of an *R. salmoninarum* infection was observed in Chinook salmon collected from BRW, followed by LMRW, SRW, and MCW (Figure 5.2A). The combined *R. salmoninarum* prevalence in females from all four weirs was nearly double that of males (8.7% vs. 4.95%); however, this was not a significant difference in prevalence ($F = 2.58$; df = 1, 654; $P = 0.1089$). More specifically, female Chinook salmon from LMRW and MCW exhibited a higher prevalence of *R. salmoninarum* than males (Figure 5.2B). Conversely, male Chinook salmon collected from BRW and SRW were found to demonstrate a higher prevalence of *R. salmoninarum* than females (Figure 5.2B).

In order to ascertain the extent to which infected fish were potentially shedding *R. salmoninarum* along with the gametes, Q-ELISA was also performed on ovarian fluid and milt

from spawning Chinook salmon returning to LMRW, MCW, and SRW (Table 5.2, Figure 5.3A). The overall prevalence of *R. salmoninarum* detected in gametes from the three weirs was 4.3% (23/540 fish). By location, potential shedding was most prevalent in Chinook salmon from SRW (6.7%), followed by LMRW (4.0%) and MCW (3.7%) (Figure 5.3A). Overall, potential shedding within the gametes of females (6.0%) was twice as high in prevalence as males (3.1%), although this was not statistically significant ($\chi^2 = 2.73$; df = 1; $P = 0.098$). The increased prevalence of *R. salmoninarum* in females was likely due to fish collected from LMRW and MCW, where females had a higher prevalence of *R. salmoninarum* in their gametes (Figure 5.3B).

To gain a more accurate perception of an individual's ability to shed the bacterium, the Q-ELISA results from the kidney and spleen sample and the gamete sample from LMRW, MCW, and SRW Chinook salmon were combined and placed into one of the four groups previously described (Figure 5.4). The majority of the fish tested were not shedding the bacterium, with *R. salmoninarum* not detected in the gametes (kid+/gam-) or in either sample (kid-/gam-) (Figure 5.4). There was also no discernible difference in shedding between the male and female Chinook salmon (Figure 5.4).

4.2. Detection of *R. salmoninarum* at LMRW

Little Manistee River Weir is the main gamete collection site for the MDNR and therefore was chosen for additional analysis.

4.2.1. Results of Q-ELISA

For this assay, Chinook salmon were collected at three time periods: before the typical spawning season ('pre-season'), early in the spawning season ('early run'), and late in the spawning season ('late run'). Both prevalence and intensity were observed to generally increase in the late run as opposed to the early run (Table 5.2, Figure 5.5A), which was also statistically significant ($\chi^2 = 6.67$; df = 1; $P = 0.01$). Additionally, the female Chinook salmon had a significantly higher prevalence of *R. salmoninarum* detected than the male individuals in the early run ($\chi^2 = 6.86$; df = 1; $P = 0.009$) (Figure 5.5B).

Comparisons of the prevalence of an *R. salmoninarum* infection within the gametes of Chinook salmon from LMRW showed a pronounced increase in the later runs compared to earlier runs (Table 5.2; Figure 5.6A). More specifically, the observed increase in prevalence from the early to late run was determined to be significant ($\chi^2 = 6.40$; df = 1; $P = 0.011$) (Figure 5.6A), which was due the increased prevalence of *R. salmoninarum* detected in the milt from infected males ($\chi^2 = 4.30$; df = 1; $P = 0.038$) (Figure 5.6B). Generally, the prevalence and intensity of the *R. salmoninarum* infection was more prominent in the female individuals, but this trend was not statistically significant ($\chi^2 = 3.16$; df = 1; $P = 0.076$) (Figure 5.6B).

4.2.2. Results of nPCR

Nested PCR was performed on early and late run samples only. The detection of *R. salmoninarum* in kidney and spleen homogenates via nPCR varied significantly according to

time of sampling (early run vs. late run) and sex. Overall, *R. salmoninarum* was detected from 23.2% of the fish sampled (85/366 fish), with 31.2% of the females (43/138 fish) and 18.4% of the males (42/228 fish) being infected (Table 5.2). Moreover, nPCR detected *R. salmoninarum* at a significantly higher rate in the late run Chinook salmon compared to the early run individuals ($\chi^2 = 16.48$; df = 1; $P < 0.001$) (Table 5.2). Additionally, nPCR detected a higher prevalence of *R. salmoninarum* in the kidneys of LMRW females than males, specifically in the early run individuals ($\chi^2 = 10.76$; df = 1; $P = 0.001$) (Table 5.2).

4.2.3. Results of bacterial culture

Bacterial culture was performed on early and late run samples only. Culture revealed the presence of *R. salmoninarum* in 11.9% of male fish (20/168 fish) and 12.9% of female fish (12/93 fish), for an overall prevalence of 12.3% (32/261 fish; Table 5.2). Colony forming units (CFUs) varied from 3-256 CFU g^{-1} of kidney tissues in early run fish (mean = 2.7 CFU g^{-1}) to 5-912 CFU g^{-1} in the late run fish (mean = 30 CFU g^{-1}). Overall, the prevalence of *R. salmoninarum* detected by culture was significantly higher in the late run Chinook salmon compared to the early run ($\chi^2 = 16.03$; df = 1; $P < 0.001$), which was due to the higher detection of *R. salmoninarum* in the male fish than female fish ($\chi^2 = 14.31$; df = 1; $P < 0.001$) (Table 5.2).

4.2.4. Detection of circulating antibodies against *R. salmoninarum* by agglutination

Agglutination was performed on early and late season samples only. The analysis revealed that ~40% of LMRW Chinook salmon were producing antibodies (105/261 fish; Table 5.2). There was a larger number of early run Chinook salmon with measurable antibodies as opposed to the late run ($\chi^2 = 18.93$; df = 1; $P < 0.001$) (Table 5.2). Additionally, male fish produced significantly more circulating antibodies than female fish ($\chi^2 = 4.92$; df = 1; $P = 0.027$), with titers varying from 2^3 to 2^{11} (Table 5.2). Of the early run Chinook salmon, the number of males with circulating antibodies were higher compared to their females cohorts ($\chi^2 = 4.26$; df = 1; $P = 0.039$) (Table 5.2).

4.3. Disease stage analysis

Grouping of CHS into potential stages of infection was performed on infected fish for which the agglutination, culture, nPCR, and Q-ELISA assays were performed, expanding on the observations of Faisal and Eissa (2009). Fish were grouped into six stages based on positive results from the assays: Stage 1 represented the onset of an infection (nPCR+, culture- , Q-ELISA-, agglutination-), Stage 2 signified a settled infection (nPCR+, culture+, Q-ELISA+/-, agglutination-), individuals in Stage 3 had an active infection (nPCR+, culture+, Q-ELISA+, agglutination-), Stage 4 represented the beginning of remission (nPCR+, culture-, Q-ELISA+, agglutination-), individuals in Stage 5 were in advanced remission (nPCR-, culture-, Q-ELISA+,

agglutination+), and Stage 6 signified fish were recovering from an infection (nPCR-, culture-, Q-ELISA-, agglutination+) (Table 5.1).

Chinook salmon from LMRW collected during the early and late run exhibited different patterns of disease stages (Figure 5.7A). Early run Chinook salmon included more individual fish that possessed circulating antibodies against *R. salmoninarum* (Stage 6) and less in all other infection stages as compared to those individuals in the late run (Figure 5.7A). Moreover, there was a substantial increase in the number of individuals in Stages 1-4 from the early to the late sampling period (Figure 5.7A). These observations were supported by the logistic regression analysis which revealed that the incidence of Chinook salmon at different stages of disease varied significantly as a function of the sampling period ($F = 4.2$; $df = 1, 152$; $P = 0.042$) and sex ($F = 5.92$; $df = 1, 152$; $P = 0.016$). There was also a significant interaction between the sex of Chinook salmon and the collection period ($F = 6.22$; $df = 1, 152$; $P = 0.014$). More specifically, female fish in the early sampling period were more likely to occur in higher disease stages than female fish in the late sampling period, with an odds ratio of 0.1437 ($t = -2.93$; $df = 152$; $P = 0.004$) (Figure 5.7B). On the contrary, there was no difference in the occurrence of males in disease stages from early to late sampling periods significant period effect for males observed ($t = -0.44$; $df = 152$; $P = 0.663$) (Figure 5.7C). In the early period, males were 5.14 times more likely to occur in a lower disease stage compared to females ($t = 3.49$; $df = 152$; $P = 0.0006$). However, no significant sex difference was observed in fish collected in the late period ($t = -0.08$; $df = 152$; $P = 0.934$). Most interestingly, analysis revealed that shedding of *R. salmoninarum* occurs primarily from fish in Stage 3.

Interestingly, when the non-infected fish (nPCR-, culture-, Q-ELISA-, agglutination-) were included in the analysis as an additional stage, there was no significant effect of sampling period, gender, or their interaction on disease stage ($0.17 < p < 0.66$).

5. Discussion

Renibacterium salmoninarum was detected from Chinook salmon collected from all of the weirs, although the intensity of the infection depended upon location, as well as the sex of the fish. Interestingly, the highest overall prevalence of *R. salmoninarum* was documented at SRW in the Lake Huron watershed; yet it has been shown that in the past decade, SRW generally has a lower rate of infection of *R. salmoninarum* than Lake Michigan weirs (Chapter 2). Starting in 2002, the MDNR adopted enhanced biosecurity measures, which have severely reduced the intensity of *R. salmoninarum* infections in state-run hatcheries and at the gamete-collecting weirs (Chapter 2). It is possible that MDNR-raised fingerlings stocked into the Lake Huron watershed in previous years had a higher rate of infection, which did not diminish while the Chinook salmon matured in Lake Huron and was maintained upon returning to spawn.

Furthermore, *R. salmoninarum* was detected in the kidney and spleen sample of female Chinook salmon at nearly double the rate of male Chinook salmon. It has been suggested that female fish play a greater role in the vertical transmission of *R. salmoninarum* than males (Evelyn et al., 1984; Evelyn et al., 1986; Bruno and Munro, 1986), but it is possible that female Chinook salmon are more susceptible to *R. salmoninarum* infection than males. Female Chinook salmon are more likely to be stressed from the spawning experience than males

(Carruth et al., 2000), which could contribute to a higher susceptibility to pathogens (e.g., *R. salmoninarum*) than males. Gamete production, migration, and spawning requires most of the energy stored in fish, thus weakening their immune system and making them more susceptible to pathogens. To fully examine this possibility, a sex susceptibility study of Chinook salmon to *R. salmoninarum* would need to be performed.

Interestingly, most of the Chinook salmon tested were not shedding *R. salmoninarum* in their gametes and it was not found in their kidneys and spleen either. *Renibacterium salmoninarum* was detected from both the kidneys and spleen *and* gametes of few fish, which were determined to be the potential shedders. Most of the fish shedding the bacterium were also experiencing an active infection (Stage 3), with viable *R. salmoninarum*, DNA, and antigen being detected. Fish occurring in Stage 3 were the most heavily infected individuals, thus increasing the likelihood that they would shed the bacterium. Due to the heavy infection, Chinook salmon in Stage 3 were also more likely to die, which would contribute to the low number of fish shedding *R. salmoninarum*.

Most surprisingly was that a small number of fish were shedding *R. salmoninarum* in their gametes, yet their kidneys and spleen were negative. It is possible that these fish have recovered systemically, yet remnants of *R. salmoninarum*, or its soluble antigens, still remained in the milt or ovarian fluid. Unlike the kidney and spleen samples, *R. salmoninarum* was detected at fairly equal rates in the gametes from male and female Chinook salmon, suggesting that while female fish may be more susceptible to an *R. salmoninarum* infection, they may play similar roles in shedding and vertical transmission of the bacterium. It has long been known that female fish transmit *R. salmoninarum* vertically to their offspring, but this brings to light

that male fish may have a greater role than previously suggested. While other studies have recorded that male fish do not significantly contribute to vertical transmission (Klontz, 1983; Evelyn et al. 1986; Pascho and Elliott, 2004), more than 3% of males in this study were capable of shedding the bacterium in their milt, which has also been observed by our earlier studies (Eissa et al., 2007; Chapter 2). Therefore, it is recommended that the role of males in shedding be further evaluated and explored. Additionally, the greatest potential for shedding of *R. salmoninarum* in gametes occurred at SRW. This is likely due to Chinook salmon from SRW also having the highest rate of *R. salmoninarum* infection in their kidneys and spleens. At SRW, most of the fish had concordance regarding the *R. salmoninarum* infection in their gametes and their kidneys and spleen.

Lastly, this study expands on the suggestion of Faisal and Eissa (2009) that discrepancy among diagnostic assay results is associated with the stage of infection that fish are experiencing. Using nPCR, bacterial culture, and Q-ELISA, the authors proposed that fish could be placed into one of six disease stages. Stage 1 was an initial infection (nPCR+, culture–, Q-ELISA–), implying that there was a minimal amount of bacteria in the tissues collected, and only the DNA of *R. salmoninarum* was detected. Stage 2 occurred once the bacteria was viable and numerous enough to be cultured on selective medium, and has become established (nPCR+, culture+, Q-ELISA–). Stage 3 occurred once the infection progressed to a systemic, well-established infection (nPCR+, culture+, Q-ELISA+). Stage 4 implied that a fish host has started to recover from an infection, with only bacterial DNA and antigen still remaining (nPCR+, culture–, Q-ELISA+). Stage 5 occurred when only low levels of bacterial antigen remained and as a result, the fish was experiencing an advanced stage of recovery (nPCR–, culture–, Q-ELISA+). Finally,

Stage 6 occurred in fish that have never been exposed to *R. salmoninarum*, were resistant to infection, or have eliminated the infection from their systems (nPCR–, culture–, Q-ELISA–). While Faisal and Eissa (2009) laid the groundwork with this novel explanation of discrepancies among assays, this study expands on the concept by incorporating a fourth assay, agglutination. The addition of the agglutination assay allows for researchers to gain a greater understanding of the stages of infection, as it detects the production of circulating antibodies in a fish host. A higher amount of antibodies being produced by a fish host is indicative of a recently occurring or cleared infection, while lower amounts of antibodies implies that a fish has not been recently infected. Additionally, it was decided to exclude non-infected fish from this analysis. At the time of sampling in 2005, the prevalence of BKD was declining in salmonid species returning to spawn at several Lake Michigan weirs, from a prevalence of 100% and 82% in 2001 and 2002 respectively, to 33% in 2005 (Chapter 2). As it was expected that several of the fish would not be infected due to the declining prevalence, they would not provide worthwhile information to determining the stages of infection and were omitted.

Moreover, to investigate potential disease progression over time, the present study also assessed the occurrence of disease stages in LMRW Chinook salmon at two time points. A general trend was observed of earlier disease stages, and more intense infections, occurring in the late run Chinook salmon compared to the early run. The majority of the early run fish were producing circulating antibodies and did not have an active *R. salmoninarum* infection. Furthermore, microbiological culture revealed that the intensity of the infections from early run fish was also less, as demonstrated by a lower average number of CFUs recovered from the early run Chinook salmon. On the contrary, the higher average number of CFUs from the late

run fish, along with a greater detection of *R. salmoninarum* antigen and DNA, indicates a more intense, active infection. It is possible that the early run Chinook salmon were healthier than fish in the later run, enabling them to return to spawn earlier. Chinook salmon that returned to spawn in the late sampling period exhibited signs of increased settled and active infections, which could have delayed their spawning migration. This is not entirely unexpected as fish are likely to become more stressed and susceptible to disease as their spawning run continues.

Alternatively, the individuals that returned to spawn during the early sampling period may have had a genetic advantage. While salmon have evolved to spawn at times that are ideal based on temperature regimes and other environmental factors, it has also been suggested that salmon are spawning at earlier times as an indirect result of hatchery practices (Quinn et al., 2002). Factors that would typically select against early spawning are relaxed in a hatchery environment, where there is ample food availability and protection from predators (Quinn et al., 2002). Moreover, offspring from late spawners might not reach the required size in an appropriate period of time, resulting in selective culling or low survival rates after stocked (Quinn et al., 2002). Thus, the early run LMRW Chinook salmon may have a better fitness than the late run individuals, resulting in a heightened resistance to disease as well.

As the LMRW is the main source of gametes for state fish hatcheries in Michigan, it is evident that the time of gamete harvest can contribute to the *R. salmoninarum* load in donor fish, as well as in the potential shedding. Agencies should consider the time period at which gametes are harvested, as this can affect the rate of *R. salmoninarum* infection being introduced into the hatchery system. Additionally, findings from this study suggests that using a single diagnostic assay to detect, and consequently cull, spawning CHS is not enough since

each of these assays measures a different target of the bacterium; however, performing all four assays is an important way to evaluate the progress of *R. salmoninarum* infection in a particular stock.

APPENDIX

Table 5.1. Diagnostic disease stages were determined based on *R. salmoninarum* recovered from a kidney and spleen homogenate and the presence of circulating antibodies as detected by agglutination. Nested polymerase chain reaction (nPCR), culture, semi-quantitative enzyme-linked immunosorbent assay (Q-ELISA), and agglutination (Agglut.) were used to determine disease stages.

Disease Stage	Methods of Detection[1]				Potential explanation
	nPCR	Culture	Q-ELISA	Agglut.	
1	P	N	N	N	Onset of infection
2	P	P	P/N	N	Settled infection
3	P	P	P	N	Active infection and potential mortality
4	P	N	P (low)	N	Beginning of remission
5	N	N	P	P	Advanced remission
6	N	N	N	P	Recovery

[1] P and N refer to positive and negative evidence for infection detected.

Table 5.2. The prevalence of *Renibacterium salmoninarum* detected in the blood, a kidney and spleen homogenate, and reproductive fluids from male (♂) and female (♀) Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn at the Little Manistee River Weir. Detection of *R. salmoninarum* as circulating antibodies in the blood was determined by an agglutination assay (Agglut.), while the prevalence of *R. salmoninarum* in the kidney and spleen homogenate (k/s) was detected by bacterial culture, nested polymerase chain reaction (nPCR), and semi-quantitative enzyme-linked immunosorbent assay (Q-ELISA). Occurrence of *R. salmoninarum* in the reproductive fluids (gam) was also determined by Q-ELISA. ND = not done.

Assay	Male				Female				Combined			
	Pre-Season	Early	Late	Total	Pre-Season	Early	Late	Total	Pre-Season	Early	Late	Overall
Agglut.	ND	55.8% (63/113)	23.6% (13/55)	45.2% (76/168)	ND	38.5% (20/52)	22% (9/41)	31.2% (29/93)	ND	50.3% (83/165)	22.9% (22/96)	40.2% (105/261)
Culture	ND	5.3% (6/113)	25.5% (14/55)	11.9% (20/168)	ND	7.7% (4/52)	19.5% (8/41)	12.9% (12/93)	ND	6.1% (10/165)	22.9% (22/96)	12.3% (32/261)
nPCR	ND	12.1% (21/173)	38.2% (21/55)	18.4% (42/228)	ND	28.1% (27/96)	38.1% (16/42)	31.2% (43/138)	ND	17.8% (48/269)	38.1% (37/97)	23.2% (85/366)
Q-ELISA (k/s)	0% (0/30)	1.7% (3/173)	9.1% (5/55)	3.5% (8/228)	10% (3/30)	8.3% (8/96)	14.3% (6/42)	10.1% (14/138)	5% (3/60)	4.1% (11/269)	11.3% (11/97)	6.0% (22/366)
Q-ELISA (gam)	0% (0/30)	1.7% (3/173)	7.3% (4/55)	3.0% (7/228)	0% (0/30)	5.2% (5/96)	11.9% (5/42)	7.2% (10/138)	0% (0/60)	3.0% (8/269)	9.3% (9/97)	4.6% (17/366)

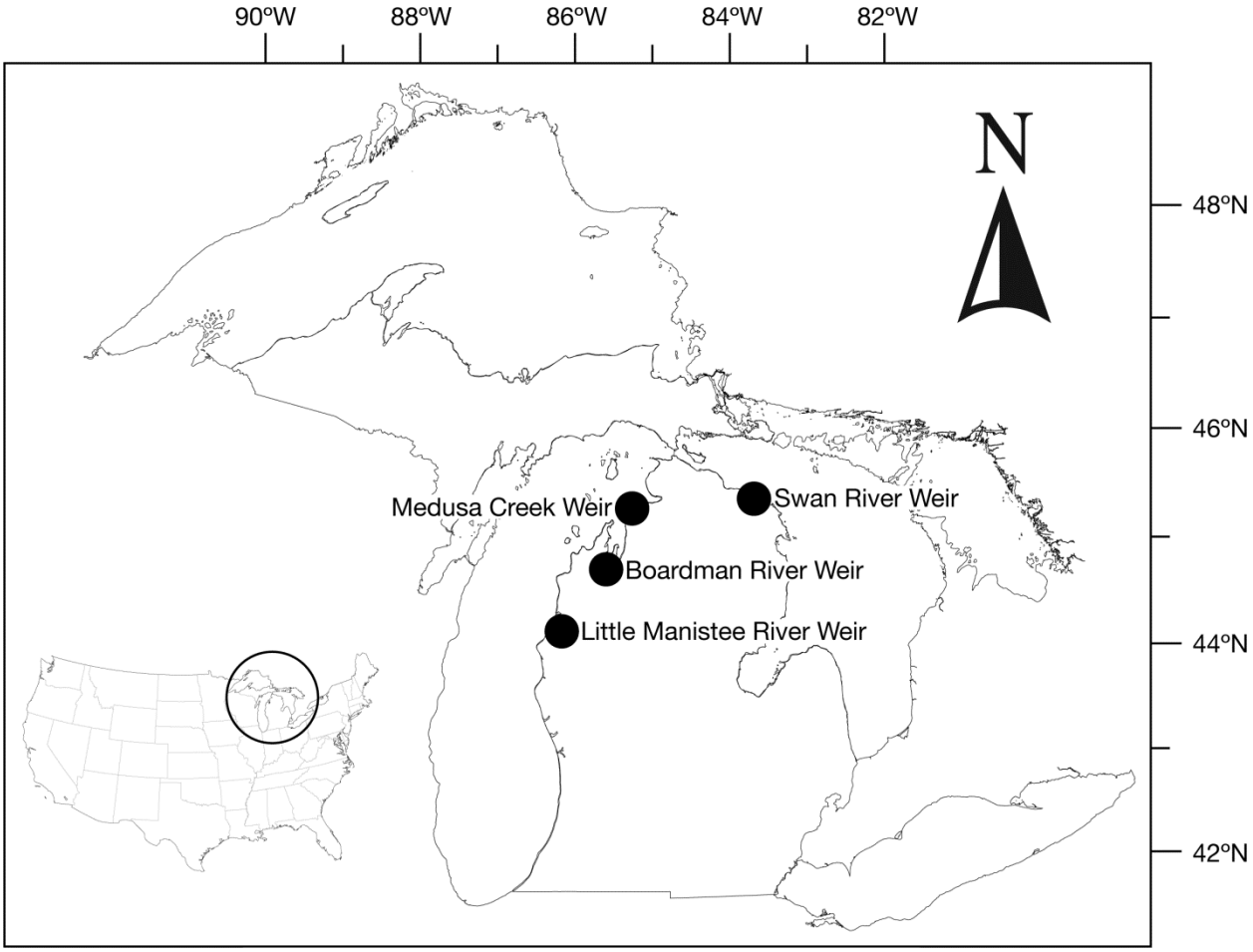

Figure 5.1. The Michigan Department of Natural Resources gamete-collecting weirs where Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn were collected in September and October of 2005: Little Manistee River Weir, Medusa Creek Weir, and Boardman River Weir (Lake Michigan watershed), and Swan River Weir (Lake Huron watershed).

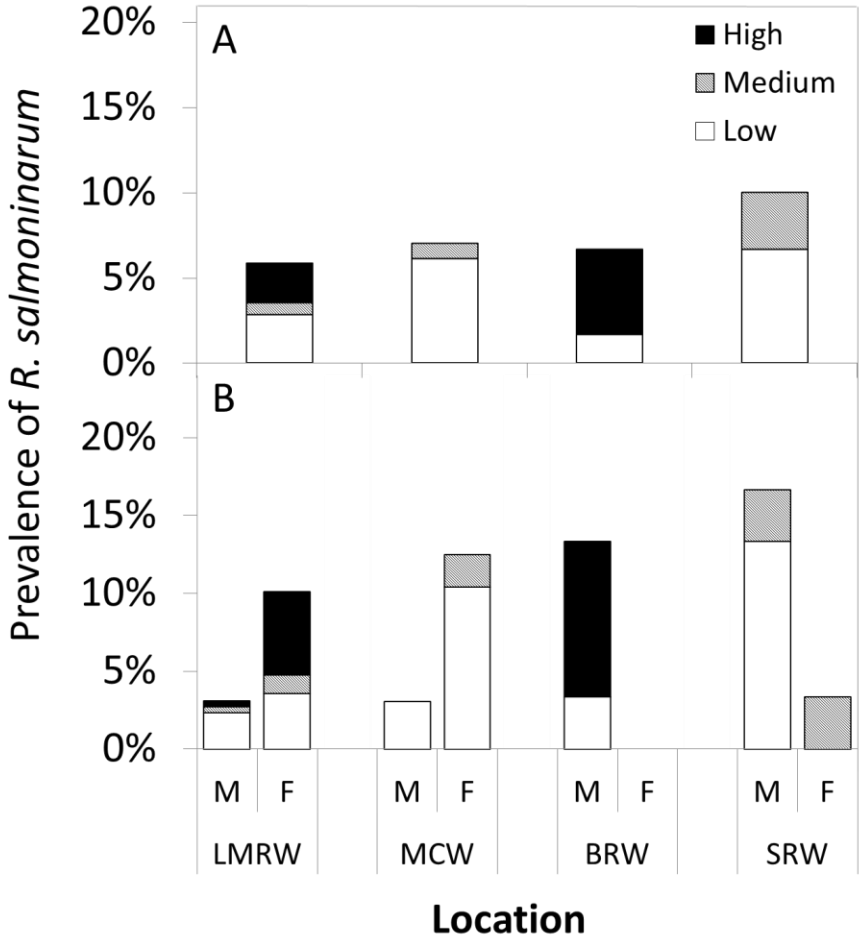

Figure 5.2. The prevalence and intensity (high, medium, low) of *Renibacterium salmoninarum* detected by the quantitative enzyme-linked immunosorbent assay in a kidney and spleen homogenate from A) all Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn at Little Manistee River Weir (LMRW), Medusa Creek Weir (MCW), Boardman River Weir (BRW), and the Swan River Weir (SRW), and B) male (M) and female (F) Chinook salmon returning to spawn at LMRW, MCW, BRW, and SRW.

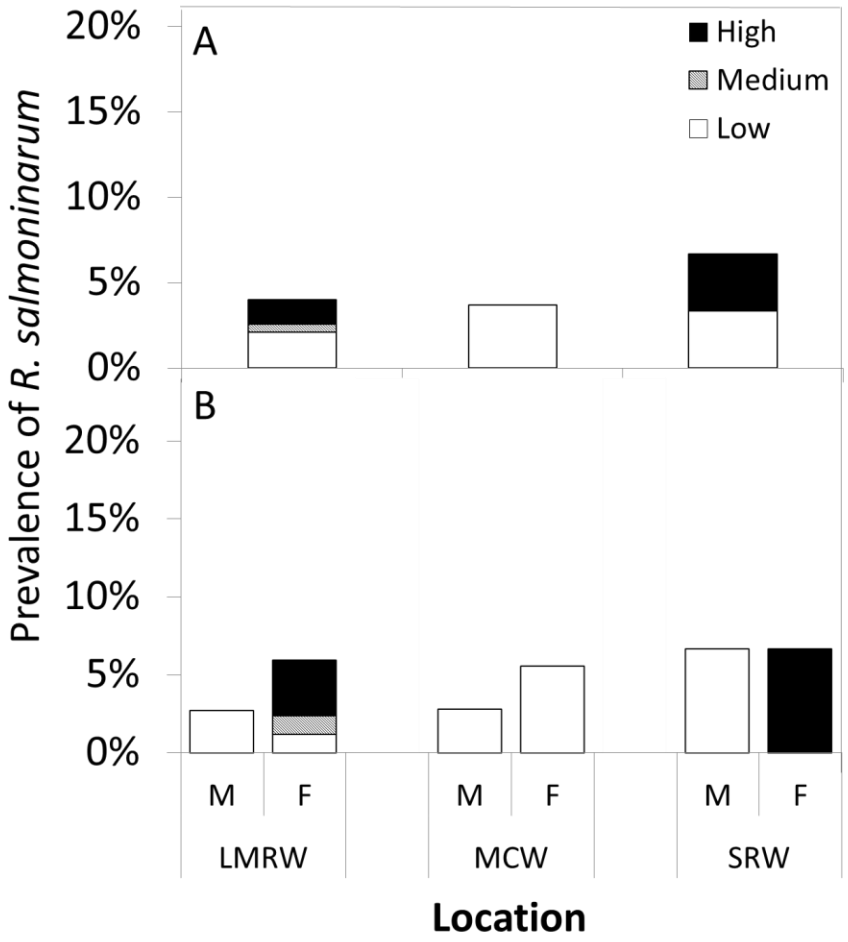

Figure 5.3. The prevalence and intensity (high, medium, low) of *Renibacterium salmoninarum* detected by the quantitative enzyme-linked immunosorbent assay in reproductive fluids from A) all Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn at Little Manistee River Weir (LMRW), Medusa Creek Weir (MCW), and the Swan River Weir (SRW), and B) male (M) and female (F) Chinook salmon returning to spawn at LMRW, MCW, and SRW.

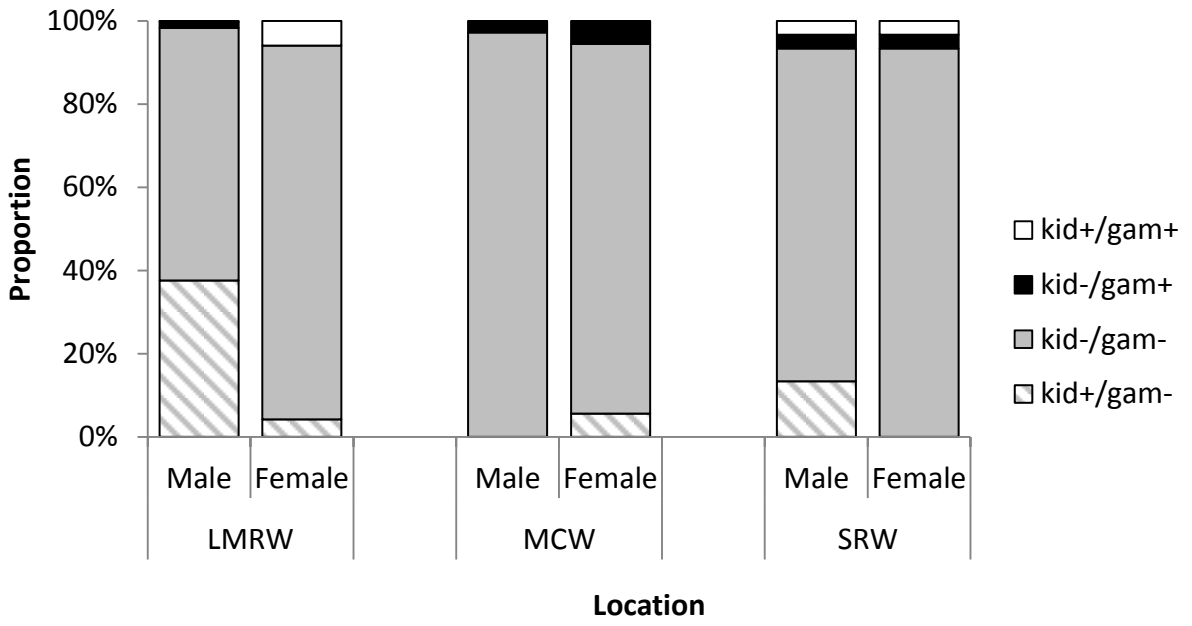

Figure 5.4. The proportion of male and female Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn at the Little Manistee River Weir (LMRW), Medusa Creek Weir (MCW), and Swan River Weir (SRW) that may be shedding *Renibacterium salmoninarum* in their reproductive fluids (kid+/gam+, kid-/gam+) and are not shedding *R. salmoninarum* (kid-/gam-, kid+/gam-), as detected by the quantitative enzyme-linked immunosorbent assay.

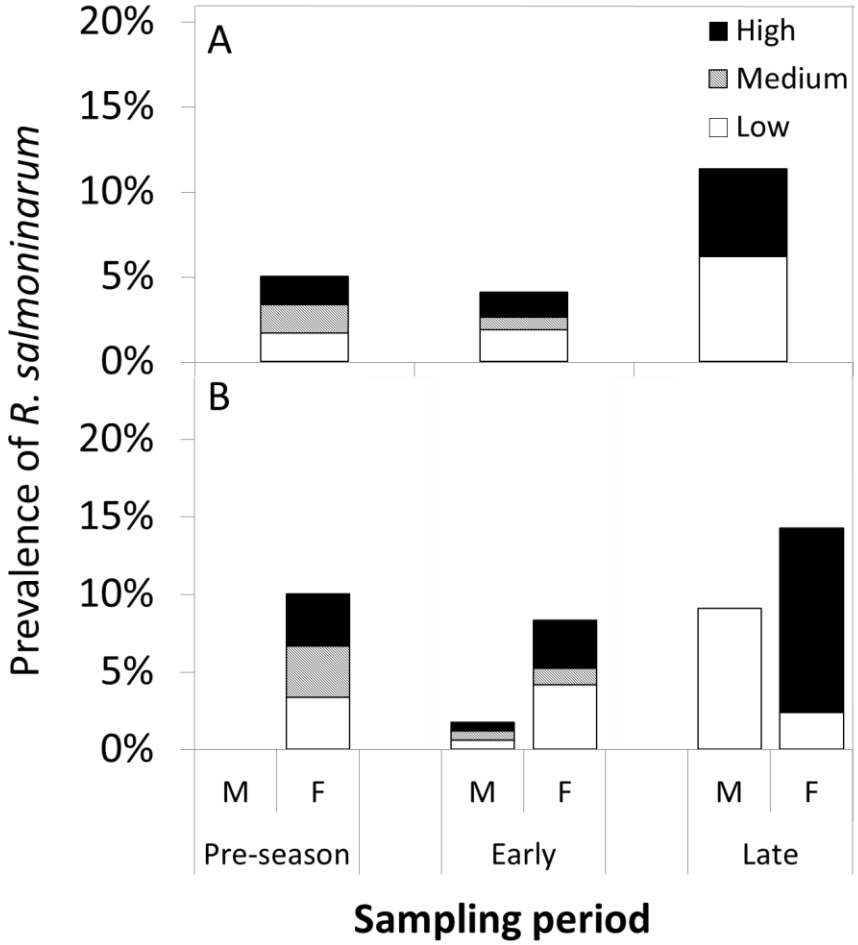

Figure 5.5. The prevalence and intensity (high, medium, low) of *Renibacterium salmoninarum* detected by the quantitative enzyme-linked immunosorbent assay in a kidney and spleen homogenate from A) all Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn at the Little Manistee River Weir during the pre-season, early, and late sampling periods, and B) male (M) and female (F) Chinook salmon during the same sampling periods.

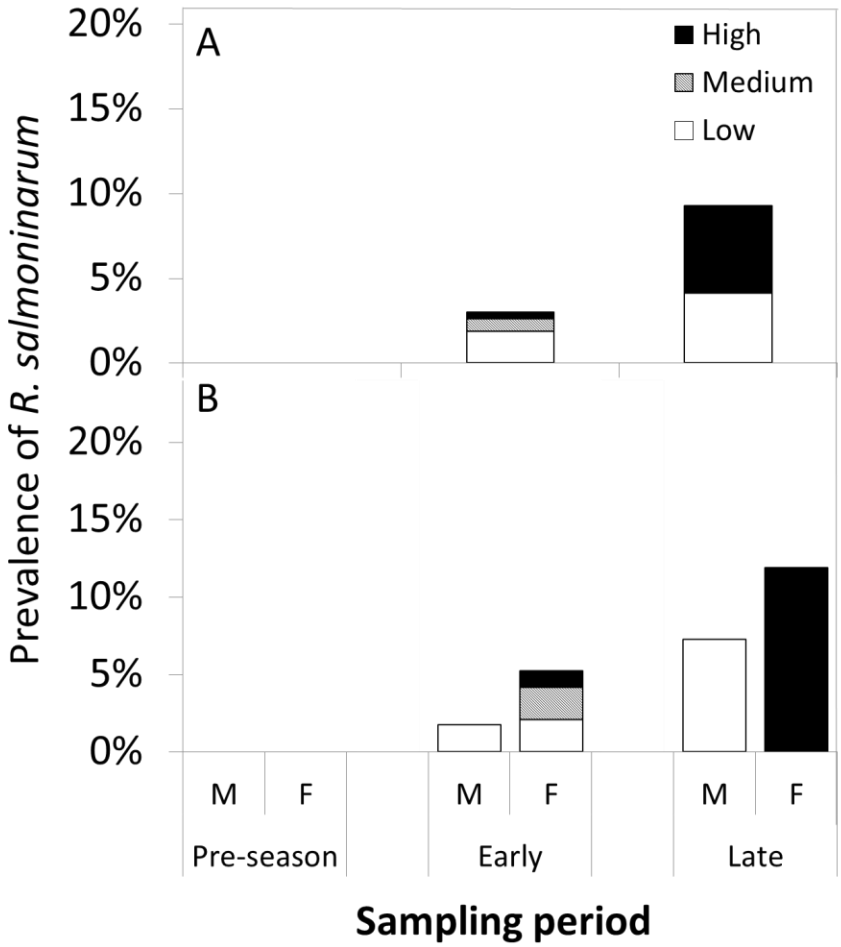

Figure 5.6. The prevalence and intensity (high, medium, low) of *Renibacterium salmoninarum* detected by the quantitative enzyme-linked immunosorbent assay in reproductive fluids from A) all Chinook salmon (*Oncorhynchus tshawytscha*) returning to spawn at the Little Manistee River Weir during the pre-season, early, and late sampling periods, and B) male (M) and female (F) Chinook salmon during the same sampling periods.

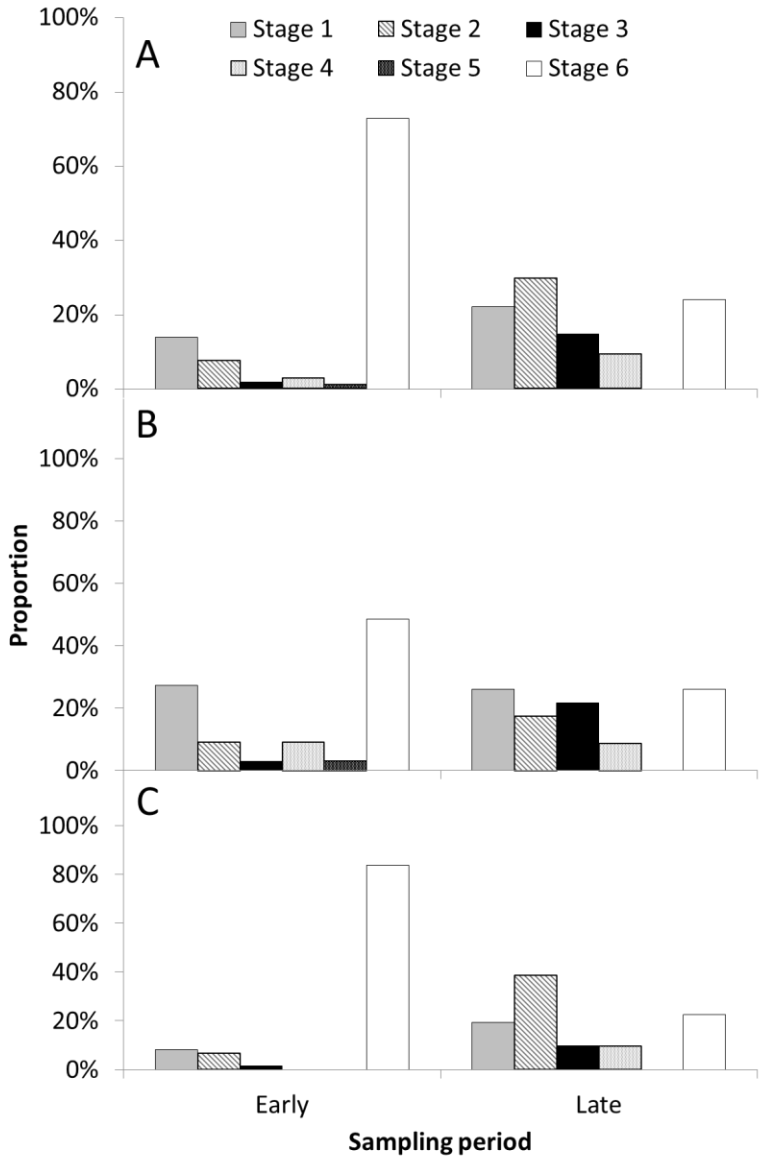

Figure 5.7. Proportion of Chinook salmon (*Oncorhynchus tshawytscha*) collected from Little Manistee River Weir in each disease stage (1-6) during the early and late sampling periods, including A) all Chinook salmon, B) female Chinook salmon, and C) male Chinook salmon. Disease stages were determined based on results from nested polymerase chain reaction (nPCR), bacterial culture, semi-quantitative enzyme-linked immunosorbent assay (Q-ELISA), and agglutination (Agglut). Stage 1 represents the onset of infection (nPCR+, culture-, Q-ELISA-, Agglut-), stage 2 represents a settled infection (nPCR+, culture+, Q-ELISA+/-, Agglut-), stage 3 represents an active infection (nPCR+, culture+, Q-ELISA+, Agglut-), stage 4 represents the beginning of remission (nPCR+, culture-, Q-ELISA+, Agglut-), stage 5 represents advanced remission (nPCR-, culture-, Q-ELISA+, Agglut+), and stage 6 represents recovering from an infection (nPCR-, culture-, Q-ELISA-, Agglut+).

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Chapter 6

Conclusions and Future Research

1. Conclusions

Considerable research has shown that the incidence of Bacterial Kidney Disease (BKD) in feral and propagated salmonid populations can be reduced and further controlled by employing several methods, including broodstock screening and culling, regular health inspections, and biosecurity measures at fish-rearing facilities. However, diagnosis of BKD can be inconsistent and greatly depends on the assay being used and the purpose of testing. In the Great Lakes basin (GLB), the prevalence of BKD has substantially declined over the past 10 years in Chinook salmon, coho salmon, and rainbow trout populations, making it even more imperative to continue to maintain the health of these economically and ecologically important populations. Continuing to monitor and control BKD infections, as well as developing new testing methods to enhance the detection of *Renibacterium salmoninarum*, is vital to maintain the health of the fish populations. The research presented in this dissertation emphasizes that there is still much to learn regarding the detection of BKD.

In Chapter Two, I describe how the prevalence of BKD has substantially declined over the past decade in *Oncorhynchus* spp. in the GLB. Historically, BKD has been associated with several mortality events of *Oncorhynchus* spp. in the GLB, and is known to cause chronic infections and mortalities in propagated and wild salmonids worldwide. I have shown how a drastic decline in the prevalence of BKD in wild and propagated Chinook salmon (*Oncorhynchus tshawytscha*), coho salmon (*O. kisutch*), and steelhead (*O. mykiss*) has coincided with the implementation of enhanced biosecurity measures at Michigan Department of Natural Resources (MDNR) gamete-collection weirs and hatchery facilities. It was found that

biosecurity measures, such as selective culling, egg disinfection, separate nets for raceways, disinfecting footbaths, and regular screening of broodstock and pre-stocking fish, are important mechanisms for preventing and controlling the spread of *R. salmoninarum* in wild and enclosed environments. Over the ten-year period, it was also discovered that prevalence was typically higher in Chinook salmon from the Lake Michigan watershed than the Lake Huron watershed, which is also where BKD-associated large-scale mortalities have occurred in the past.

Within Chapter Three, I investigated the effects of exposure route and resultant disease course on the ability of non-lethally collected samples (blood, mucus, and a urine/feces mixture) to detect *R. salmoninarum* compared to the currently accepted method of a lethally collected kidney and spleen sample. While a urine/feces mixture was determined to have the greatest potential as an acceptable non-lethal surrogate, testing both a urine/feces mixture and a kidney and spleen sample was the best method for detecting *R. salmoninarum* prevalence and intensity in a population. This would greatly enhance the accuracy of disease detection, which would provide hatchery biologists with more reliable information to base their management decisions on.

In Chapter Four, I tested a single-dilution indirect enzyme-linked immunosorbent assay with the purpose of detecting anti-*R. salmoninarum* antibodies in experimentally infected juvenile rainbow trout and feral adult *Oncorhynchus* spp. The protocol used in this study was able to successfully detect antibodies in both the experimentally infected and feral fish. The antibody response observed in the experimental fish appeared to be of a short duration and did not confer protection when naïve fish were challenged with live *R. salmoninarum*. However, the antibody response of the feral fish was much higher, and also occurred in the absence of an

active *R. salmoninarum* infection in the kidneys and spleen of the fish. It is likely that the feral fish are being exposed to *R. salmoninarum* on multiple occurrences, resulting in the continuous production of antibodies. This assay could be applied as a non-invasive method to determine if captive fish are being exposed to live *R. salmoninarum*.

Within Chapter Five, I assessed the prevalence of BKD in male and female Chinook salmon returning to spawn at four MDNR gamete-collection weirs using multiple diagnostic assays, as well as the occurrence of several stages of disease. Not only was there a higher prevalence of BKD at the Swan River Weir, located in the Lake Huron watershed, but female Chinook salmon appeared to be more susceptible to *R. salmoninarum* infection than male Chinook salmon. While a higher prevalence of BKD in the Lake Huron watershed is contradictory to what was observed in Chapter 2, sampling for this study only occurred during 2005, which is encompassed by the study in Chapter 2, which transpired from 2001 to 2010. At the Little Manistee River Weir in the Lake Michigan watershed, there was also evidence of disease progression, with more intense infections documented later in the spawning period rather than earlier. This is suggestive of healthier, better fit fish returning to spawn at earlier time points, while Chinook salmon returning to spawn late in the season are more heavily infected, resulting in a slower migration. As a result, it would likely be beneficial for fishery managers to consider harvesting gametes used for propagation earlier rather than later, to reduce the influx of *R. salmoninarum* being introduced into hatcheries.

2. Future Research

The results presented in this dissertation have contributed substantially to further our understanding of *R. salmoninarum* epidemiology; particularly how the choice of different tissues for sampling and diagnostic assays can impact the detection and spread of BKD in *Oncorhynchus* spp. in the Great Lakes basin. Furthermore, it has helped to establish an important foundation for non-lethal sampling and for deciding the appropriate diagnostic assay. However, much more work is needed to ensure that the prevalence of BKD in GLB *Oncorhynchus* spp. continues to remain low.

In particular, the prevalence and intensity of BKD should continue to be recorded on an annual basis, as any increases in the presence of BKD would be observed. Also, while it is very likely that the enhanced biosecurity measures implemented by the MDNR in 2002 were the leading reason as to why the prevalence of BKD declined so substantially, this should be validated in a controlled environment. A 'control' hatchery, or one without the implemented biosecurity measures, was not utilized as the MDNR could not manage to withhold the disease prevention methods from one of the hatcheries or gamete-collection weirs, which will likely continue to be a concern. Therefore, an alternative would be to mimic these conditions in an experimental challenge. This would aid in the determination of the true effectiveness of the enhanced biosecurity measures.

Additionally, while this research evaluated the relationship between the prevalence of *R. salmoninarum* in broodstock and their resultant progeny, it would be meaningful to re-evaluate the same data, investigating the role of the wild environment on BKD infections in fish

returning to spawn at the gamete-collection weirs. Supposing that fingerlings stocked into the GLB would return to spawn in 2-3 years, the prevalence and intensity of BKD in hatchery-raised pre-stocking fingerlings would be compared to adult fish returning to spawn 2-3 years later, with the assumption that these fish would be the same cohort. This analysis could allow for us to gain a better understanding of whether or not stocked hatchery fish maintain low BKD infections while in the wild environment. Furthermore, it would be beneficial to monitor the prevalence in one- and two-year fish to further investigate the role of the natural environment in BKD infections.

Of all the experiments performed in this dissertation, the concept of using non-lethal samples as an alternative for lethal sampling has recently become much more prevalent in the scientific community. The benefits of non-lethal sampling are numerous, but most important is that valuable fish species would not have to be sacrificed for disease testing. While Chapter 4 did reveal that *R. salmoninarum* is detectable in non-lethally collected samples, future research should focus on mimicking a more natural challenge environment through the use of a cohabitation challenge. It was evident that the exposure route and infectious dose affected the detection capabilities of the tissues; therefore, cohabitation challenges with varying infectious doses would reflect a more natural infection and would give a more accurate representation of the efficacy of non-lethal samples. Also, although it can be costly, I would also recommend collecting samples more frequently, especially in a chronic disease course. Non-lethal samples collected from the chronic disease course challenge did not detect substantial levels of *R. salmoninarum*, which could be due to the timing of sample collection. If sampling occurred

more frequently, and if the study was extended for a longer period of time, we could increase the likelihood of detecting *R. salmoninarum* in tissues with low detection levels.

The indirect ELISA used in this dissertation to detect anti-*R. salmoninarum* antibodies is an efficient test for screening *Oncorhynchus* spp. fish sera. Further research should focus on optimizing the current assay and investigating its use in other salmonid species. It would be beneficial to evaluate using *R. salmoninarum* with the p57 antigen removed as a coating preparation, as this antigen can sometimes mask the detection and quantitation of fish antibodies directed against the bacteria themselves. Additionally, the feral fish that were tested in this study were a sub-sample of the available sera for antibody detection. A more thorough analysis of yearly serum samples from *Oncorhynchus* spp. during 2006 to 2013 would contribute to the current knowledge of BKD prevalence. Furthermore, as Chinook salmon from the Little Manistee River Weir (LMRW) produced the highest levels of circulating anti-*R. salmoninarum* antibodies, Chinook salmon from the Swan River Weir in the Lake Huron watershed should also be evaluated for antibodies against *R. salmoninarum*. The Swan River Weir generally has a lower incidence of BKD in Chinook salmon returning to the weir to spawn than LMRW, and as such, a lower antibody response could be expected as well.

It would also be beneficial to continue to examine the progression of disease through the use of multiple diagnostic assays, as this can influence management decisions. The study performed in Chinook salmon from the Little Manistee River Weir should be replicated over several consecutive years with more frequent sampling periods, to confirm the existence of the stages, as well as to better visualize trends in disease progression as only two time points were analyzed in this study. More complete knowledge of disease progression could influence when

the MDNR collects gametes from fish returning to spawn, as it would be advantageous to them to collect gametes during the time period when fish are least infected. As a result, this could reduce the amount of *R. salmoninarum* being brought into hatcheries through vertical transmission. Additionally, this analysis should be conducted at the other MDNR gamete-collection weirs in the GLB, as they are likely to experience different rates of BKD infection.