Identification of MH Class IIβ alleles conferring resistance/susceptibility to *Flavobacterium psychrophilum* in *Oncorhynchus mykiss* families

by

Calvin Kellendonk

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Flavobacterium psychrophilum is the causative agent of bacterial cold water disease (BCWD), which is a major concern for aquaculture and fish health, with mortality rates of 20-30%. Without a commercially approved vaccine, alternative methods of control are needed. One method involves selective breeding to produce fish with a higher natural level of resistance to BCWD. Major histocompatibility (MH) genes encode cellular receptors involved in antigen presentation and play an important role in adaptive immunity. MH genes are highly polymorphic and previous research has identified specific alleles of class I and class II that are associated with resistance/susceptibility to infectious salmon anemia virus (ISAv) as well as *Aeromonas salmonicida* in Atlantic salmon. This study examines the MH class IIβ genotype of six rainbow trout families infected with a pathogenic strain of *F. psychrophilum* and survival status to identify alleles associated with better or worse performance. In the survival analysis hazard ratios were calculated for each allele and genotype. None of the obtained alleles or genotypes demonstrated statistically significant hazard ratios. If MH class IIβ alleles did confer resistance/susceptibility to BCWD this would be valuable information for aquaculture businesses that want to use selective breeding to produce fish stocks with a reduced chance of succumbing to BCWD. However no statistically significant relationship between alleles or genotypes and resistance to BCWD was observed.

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Chapter 1

Introduction

1.1 Aquaculture in Canada

Aquaculture by definition, is the farming of fish, shellfish and aquatic plants in fresh or saltwater (Canadian Department of Fisheries and Oceans, 2013). In Canada, finfish culture was first used as a means to replenish natural stocks. However, that changed in 1970 when it evolved in response to the local and global demand for fish as a food source. Historically, fish caught from the wild had been used to meet this demand, but over time the amount of wild fish being caught decreased. The demand for fish remained steady and out of necessity the aquaculture industry was born, starting with trout and oysters.

To this day, fish remain an important food source for humans. Canada's aquaculture industry is diverse, with species farmed varying by region. Species such as *Oncorhynchus mykiss* and *Salmo salar* have become well established in aquaculture, while farming of other species are still in the development stages. There has been significant growth over the past 40 years and the industry has turned into an important contributor to the Canadian economy. Between 2000 and 2010, the annual economic contribution of the industry increased from $ 609 million to $ 927 million; almost a 50% increase in just the last decade. Today, the value of total aquaculture production in Canada is estimated to be $ 1 billion (Canadian Department of Fisheries and Oceans, 2013).

In addition to its economic contributions, aquaculture also provides employment for many Canadians. In 2009 it was estimated that the industry provides 14,000 full time jobs,

mostly in rural communities and coastal areas. Hatcheries produce employment opportunities in three main areas: 1) operation and maintenance of farms or hatcheries where fish are grown, 2) goods and services industry that supports facility operations by providing equipment and feed, 3) community employment in locations surrounding an aquaculture facility, mainly service jobs such as retail work at local grocery stores and gas stations (Canadian Department of Fisheries and Oceans, 2013). Overall, the combined contributions to the economy and employment opportunities make aquaculture a valuable industry in Canada.

1.1.1 *Oncorhynchus mykiss* in Aquaculture

Oncorhynchus mykiss, also commonly referred to as rainbow trout, are native to the North Pacific Ocean and fresh water streams of Canada and the United States (K. Hershberger, 1992). In addition to wild populations, rainbow trout are raised on inland farms for human consumption. In Canada, trout account for 3.4% of total aquaculture production. The total trout production is valued around $ 36.7 million with the majority of rainbow trout being produced in Ontario and Quebec (Canadian Department of Fisheries and Oceans, 2013). Most recently, trout production in Ontario has been valued at $ 18.3 million annually (Canadian Department of Fisheries and Oceans, 2013). In addition to its economic contribution and use as a food source, rainbow trout aquaculture also creates a plethora of employment opportunities at the local, provincial and federal levels. Therefore, monitoring fish health and maintaining facility conditions to minimize the occurrence of disease and loss from outbreaks is essential for a sustainable aquaculture industry.

1.1.2 Disease in Aquaculture

In aquaculture facilities, fish are commonly held at much higher densities than they would experience in the wild. At these high stocking densities, increased occurrence of disease and infection are observed (Barman, Nen, Mandal, & Kumar, 2013; Wildgoose, Brown, & Thresher, 2000). For aquaculture to be sustainable, disease prevention and control are critical (Barman et al., 2013; Gudding & Van Muiswinkel, 2013; Wildgoose et al., 2000). There are numerous approaches to controlling disease, ranging from well established methods to more experimental techniques.

Vaccination and antibiotic treatment are two of the older, still existing methods used to prevent and manage disease, although overuse of antibiotics is a problem that could result in resistant bacterial strains emerging (Cabello et al., 2013; Gudding & Van Muiswinkel, 2013; Hesami et al., 2010). Resistance to one particular drug limits the number of available antibiotic treatments and resistance to more than one could even eliminate chemoprophylaxis treatment options all together (Cabello et al., 2013; Defoirdt, Sorgeloos, & Bossier, 2011; Schmidt, Bruun, Dalsgaard, & Larsen, 2000). As a result, alternative methods are being investigated. These include the use of immunostimulants, novel antimicrobial peptides and probiotics (Barman et al., 2013; Cabello et al., 2013; Defoirdt et al., 2011; Wildgoose et al., 2000). Other research is examining the potential of bacteriophage treatment as a method to target specific pathogenic bacteria. More novel work explores inhibiting pathogenic bacterial growth with short-chain fatty acids or inhibiting virulence gene expression (Defoirdt et al., 2011). Lastly, genetic improvement of fish stocks to increase resistance and survivorship has been attempted (Henryon et al., 2005; Leeds et al., 2010; Wildgoose et al., 2000).

1.1.3 Bacterial Cold Water Disease

Flavobacterium psychrophilum is a psychrophilic gram-negative bacterium that causes bacterial cold water disease (BCWD). Psychrophilic bacteria can grow and survive in cold temperatures ranging from -20ºC to +10ºC. It was first reported in North America during the 1940's and later observed in Germany and France. The disease affects fish across the globe, with infections occurring throughout Asia, Europe and North America (Starliper, 2011; Virginia & Holt, 2005). Although infection is widespread, clinical presentation of the disease is highly variable. Disease presentation is dependent on the: species of fish, age of fish, strain of bacteria and geographic region. *F. psychrophilum* has a broad range of hosts, infecting both salmonids and non-salmonids. Infections in non-salmonids are less prevalent, but have been observed in carp (*Cyprinus carpio*), eel (*Anguilla anguilla*), goby (*Rhinogobius brunneus*) and perch (*Perca fluviatilis*). In contrast, infections in salmonids are more frequent, specifically in Atlantic salmon and rainbow trout (Nematollahi, Decostere, Pasmans, & Haesebrouck, 2003; Starliper, 2011; Virginia & Holt, 2005). Outbreaks most often occur in hatchery reared fish, making it primarily a disease of cultured salmonids.

In a hatchery or aquaculture grow-out facility, fish of all ages are affected by BCWD, but fry and fingerlings are by far the most susceptible to infection (L. Brown, Cox, & Levine, 1997; Starliper, 2011). Virulence of *F. psychrophilum* isolates can be highly variable and the sequencing of their genomes has provided insight on factors contributing to their pathogenicity. Adhesins, exotoxins, proteases and endotoxins are virulence factors that have all been observed to contribute to the pathogenicity of *F. psychrophilum* (Dalsgaard, 1993; Pacha, 1968; Virginia & Holt, 2005; Wiklund & Dalsgaard, 2003). Due to the bacteria's

psychrophilic nature, the regulation of its genes at cold temperature has also been examined. Several candidate genes, ranging from outer membrane proteins to RNA helicases and cassette transporters showed increased expression at low temperatures (Hesami, Metcalf, Lumsden, & Macinnes, 2011). Even after partial genome sequencing and the identification of several virulence factors, very little is known about the pathogenicity of *F. psychrophilum*.

Bacterial strain and the geographic region of infection are known to dictate the severity of disease and its presentation in fish. The most prevalent manifestation of the disease produces open lesions on the exterior of the fish and tissue necrosis at the site of colonization, usually the caudal peduncle or fin. Additional signs of disease include loss of appetite, abdominal distention and pale gills (Starliper, 2011; Virginia & Holt, 2005). Strains of *F. psychrophilum* in North America were not always the same as those found in Europe and as a result the disease manifested differently in each location. Currently North American *F. psychrophilum* infections result in acute bacterial septicemia , similar to European infections that affect early life stage fish. This acute form of the disease is often referred to as rainbow trout fry syndrome and can result in up to 50% mortality (Starliper, 2011).

In Ontario, BCWD is a disease of economical concern and a major concern in the commercial production of rainbow trout. In the past it has commonly caused tail rot, necrotic myositis and cephalic osteochondritis (Lumsden et al., 2004). More recently, fish farms are observing an acute infection resulting in bacterial septicemia. In aquaculture facilities, typical mortality rates are between 20-30%, but in severe cases can reach up to 80% (Starliper, 2011; Virginia & Holt, 2005). Currently there is no commercially approved

vaccine for BCWD, so efforts to manage the disease focus on antibiotic treatment. In Ontario, the two most commonly used treatments are oxtetracycline and florfenicol. Both are delivered orally through medicated feed and need to be administered as early as possible since loss of appetite can result from infection (Lumsden et al., 2004). As with most antibiotics, overuse and improper treatment regimes increase the risk of resistant bacterial strains emerging. For this reason, the efficiency of several antibiotics and antimicrobial compounds have been examined (Bruun, Madsen, & Dalsgaard, 2003; Rangdale, Richards, & Alderman, 1997).

Additionally, the antimicrobial susceptibility of *F. psychrophilum* isolates from Ontario has been examined to determine minimum inhibitory concentrations and the potential effectiveness of other antimicrobial agents (Hesami et al., 2010). But with no vaccine and limited treatments available, alternative methods are needed to control BCWD outbreaks.

Many of these methods focus on selection for resistance to disease at the genetic level and over multiple generations. The genetic correlation between growth and resistance to specific diseases has been investigated with variable results (Henryon et al., 2005; Silverstein et al., 2009). For example, resistance to BCWD in rainbow trout was found to be moderately heritable (0.35) and not adversely correlated with growth in one study (Silverstein et al., 2009) but weakly heritable in another study (Henryon et al., 2005). Heritability estimates are specific to a given population and environment. Variations in fish size, environment and bacterial strain can all cause differences in heritability estimates (Henryon et al., 2005; Silverstein et al., 2009). Although only moderately heritable, genetic selection for resistance to BCWD in rainbow trout is a potential method for reducing mortality rates. This potential was demonstrated by a family based selection program that

observed increased survival rates of rainbow trout infected with *F. psychrophilum*. Survival rates increased by 24.6% and 44.7% after one and two generations of selection respectively (Leeds et al., 2010).

Overall, information on using genetics to increase resistance is encouraging and supports the idea that selective breeding could be used to produce fish stocks with a higher level of resistance to BCWD. Higher levels of resistance would reduce the risk of fish succumbing to disease and decrease mortality and the associated economic losses. Research performed by Lowia Al-Hussinee at the University of Guelph examined the survival of six rainbow trout families from Lyndon Hatcheries Inc. against *F. psychrophilum* infection (Figure 1.1). These same families were examined in this thesis work.

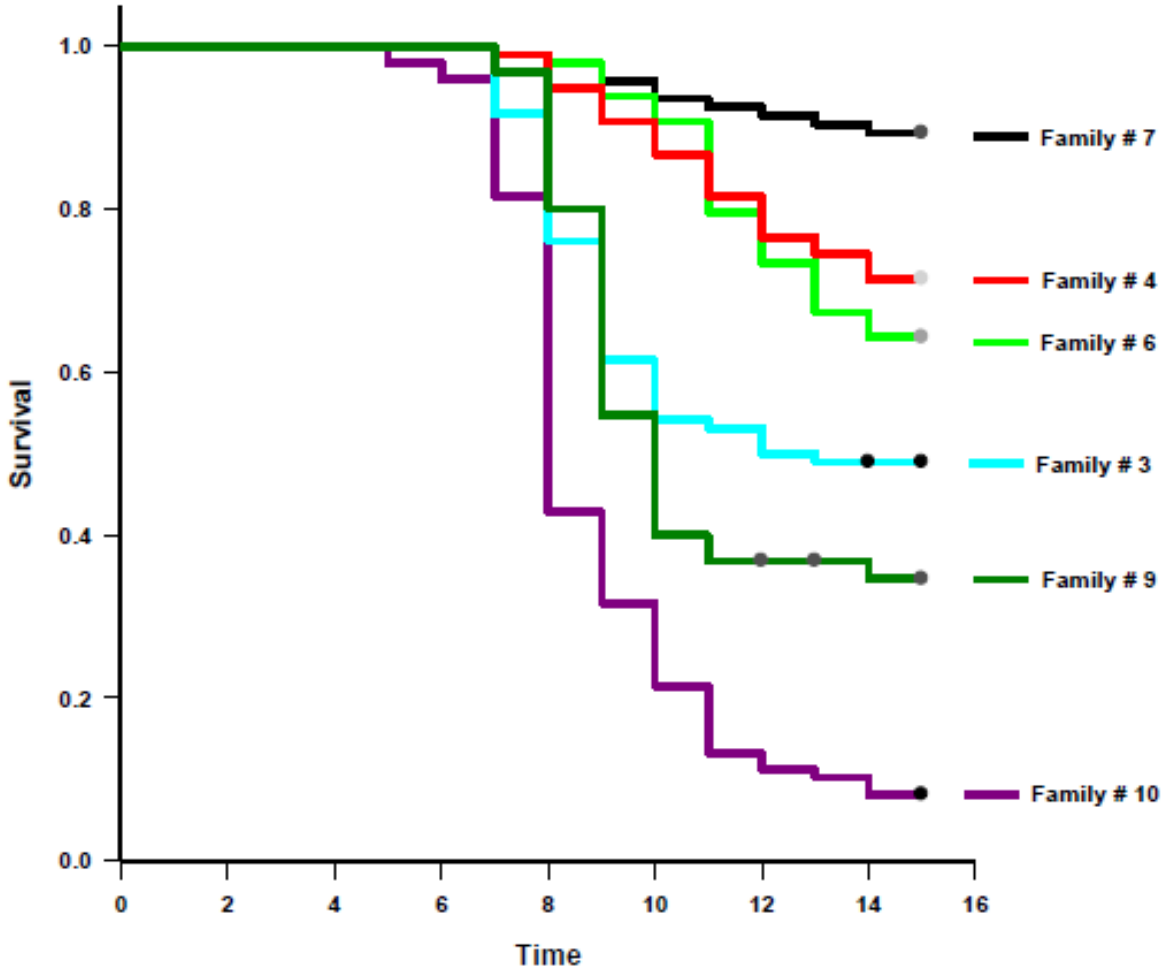

Figure 1.1 Survival plot for *Oncoryhnchus mykiss* infected by intraperitoneal injection with 3.3×10^5/0.1 mL of *F. psychrophilum* strain 101. (Figure provided by Lowia Al-Hussinee)

1.2 The Vertebrate Immune System

The immune system can be divided into two general parts; innate and adaptive immunity. Innate immunity exists in practically all multicellular animals and acts as the first line of defense against pathogens. Mechanisms of the innate immune system act non-specifically and on a wide array of organisms. In vertebrates, physical barriers, cells, antibodies and biologically active molecules such as enzymes are all important components of the innate immune system that exist prior to exposure to a pathogen (Kelley, Walter, & Trowsdale, 2005; John Trowsdale & Parham, 2004; Uribe, Folch, Enriquez, & Moran, 2011). Lysozyme for example, is an enzyme found in tears, saliva and mucous and generically attacks gram positive bacteria by hydrolyzing glycosidic bonds in the cell wall (Madigan, Martinko, Dunlap and Clark, 2006; Murphy et al., 2008). In contrast to innate immunity, adaptive immunity only exists in jawed vertebrates. And although the adaptive immune system uses components of the innate immune system, its role is quite different. The adaptive immune system provides a very specific response that is initiated upon infection. The response is selective and tailored directly to the infectious agent. Like the innate system, the adaptive system is composed of both cell and protein-mediated responses. But unlike the innate system, it has the additional aspect of pathogens specific antigen presentation (John Trowsdale & Parham, 2004).

1.2.1 Major Histocompatibility Complex

The major histocompatibility complex (MHC) genes encode cell surface receptors that have an integral role in initiating the adaptive immune response (John Trowsdale & Parham, 2004). They are the most polymorphic nuclear encoded genes known to date, with

up to 3,284 alleles being observed for the class I locus and 1,411 alleles being observed for the class II locus (Gonzalez-Galarza, Christmas, Middleton, & Jones, 2011). MHC genes are divided into class I, class II, class III, and are genetically linked in most vertebrates (Kelly et al. 2005). The linkage of these genes in birds, mice, amphibians and humans are why they are referred to as a complex (Kelley et al., 2005; J Trowsdale, 1995). To date, human and mice MHC genes have been the most extensively studied (Kelley et al., 2005) and most of the following discussion pertains to what is known about the human MHC.

Both class I and class II MHC receptors are involved in presenting peptides to T-lymphocytes and identifying what is *self* and what is *foreign* (John Trowsdale & Parham, 2004; Uribe et al., 2011). Class I receptors present intracellular peptides to $CD8^+$ T cells and can stimulate them to kill viral or intracellular bacterial infected cells (John Trowsdale & Parham, 2004; Uribe et al., 2011). The class I receptor consists of two chains; an α chain with 3 extracellular domains and a β_2 microglobulin chain (Figure 1.2). The α_1 and α_2 domains form the peptide binding groove and interact with antigens, whereas the β_2 microglobulin chain is necessary for maintaining proper receptor structure on the cell surface.

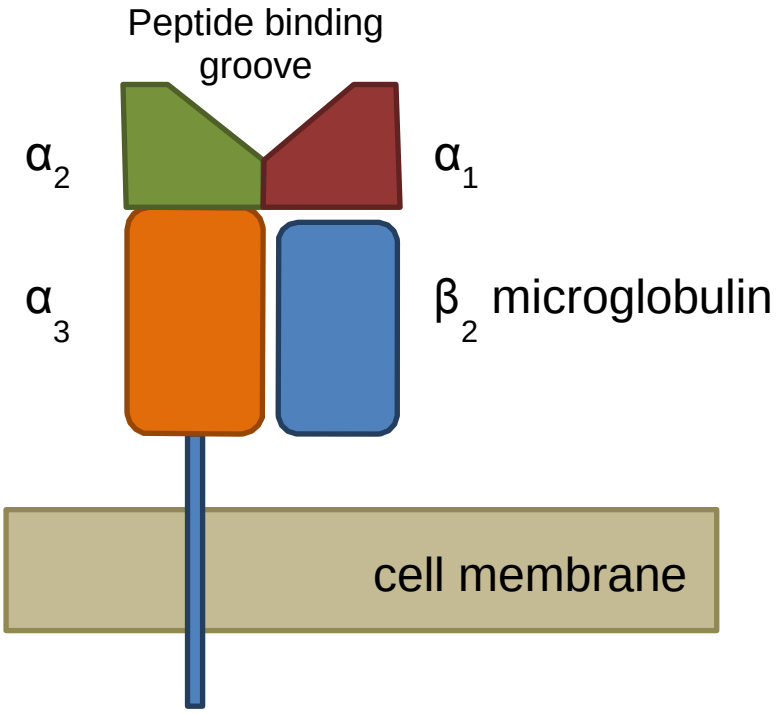

Figure 1.2 Artistic representation of MH class I receptor structure. It has three extracellular α domains plus transmembrane and cytoplasmic domain. It also includes a non-covalently bound β_2 microglobulin which has no transmembrane domain.

In contrast to class I receptors, the class II receptors present extracellular peptides to $CD4^+$ helper T cells which are involved in activating macrophages and B cells in response to extracellular pathogens like bacteria (John Trowsdale & Parham, 2004; Uribe et al., 2011). The activation of B cells leads to an antibody-mediated humoral immune response (John Trowsdale & Parham, 2004; Uribe et al., 2011). The α_1 and β_1 domains form the peptide binding groove which is open at both ends and tends to bind peptides 13-18 amino acids in length (Figure 1.3). Most of the variability between MH class I receptor allele sequences

exists in the α_1 and α_2 domains. Meanwhile most of the variability between MH class II sequences exists in the β_1 domain.

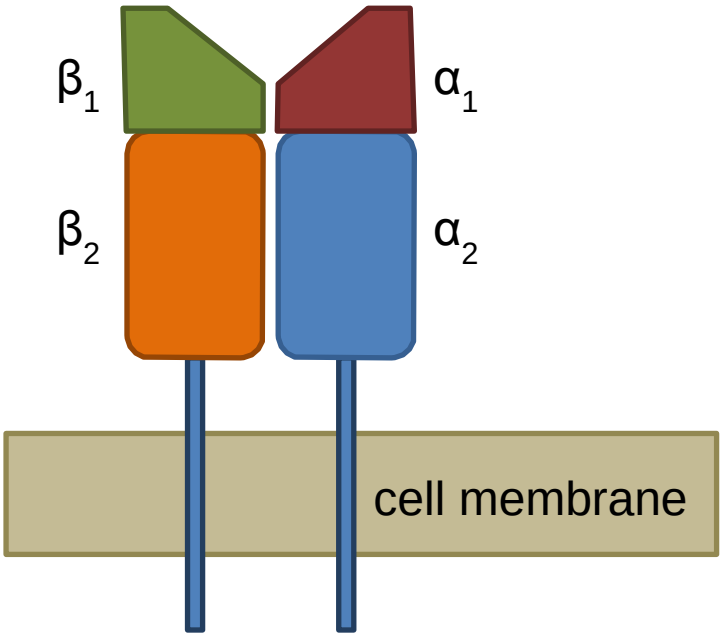

Figure 1.3 The basic structure of MH class II receptor. Both the α and β chain are anchored to the membrane with a transmembrane region and one domain from each chain forms the peptide binding groove.

1.3 Immune System of Teleost Fish

Although the amount of research on the immune system of bony fish is growing, humans and mice remain the most extensively studied. Within the group of teleost fish, examination of the immune system has been limited to a few species. These include economically important species such as rainbow trout and Atlantic salmon as well as model organisms like zebra fish (Zapata, Diez, Cejalvo, Gutiérrez-de Frías, & Cortés, 2006).

Similar to other vertebrates, fish have innate immunity composed of physical, cellular and humoral factors (Uribe et al., 2011; Whyte, 2007). Like other animals, components of the non-specific immune response are used in the adaptive response. This is demonstrated by the relationship between MH receptors and innate immunity (Dixon & Stet, 2001). Compared to humans, teleost fish have fewer classes of immunoglobulins (Uribe et al., 2011; Whyte, 2007). The main antibody of the adaptive response is IgM, but fish also possess IgD and IgT (Uribe et al., 2011; Whyte, 2007). In contrast to humans and other vertebrates, the generation of an antibody response takes much longer in fish, often up to 16 weeks to peak. Additionally, fish differ in the development of immunological memory. For T cell independent antigens only one exposure is required to produce immunological memory, whereas two exposures are needed for T cell dependent antigens (Uribe et al., 2011). T cell independent antigens can activate B cells without the assistance of T cells. In contrast, T cell dependent antigens require B and T cell interaction to produce a response. Overall the knowledge about the teleost immune system continues to grow and aid disease management in cultured fish.

1.3.1 Comparative Genomics of MHC

Even though MHC genes reside in all jawed vertebrates, their genomic organization differs by species. MHC genes have been studied in humans, non-human primates, rats, mice, marsupials, birds, reptiles, amphibians, teleost fish and chondrichthyes (Kelley et al., 2005; J Trowsdale, 1995). Independent study of these genes in a wide array of animals has allowed for comparison between and within species. Looking at these genes on such a large scale has provided insight on their rapid evolution and the force driving the changes. This has resulted

in many hypotheses regarding the reason for their rapid evolution and high level of polymorphism (Flajnik, Kasahara, & Street, 2001; Nei, Gu, & Sitnikova, 1997). One such hypothesis is that a high level of MH polymorphism within a population supports wider antigen presentation and the capability to deal with a larger array of pathogens.

Although MHC genes are usually grouped into regions with similar function (class I, II), the number of genes at each region varies between species (Flajnik et al., 2001; Kelley et al., 2005; Trowsdale, 1995). For example, humans have more than one MH class I gene clustered within the class I region, where rats have eight and frogs have one. This diversity among species can be attributed to gene duplication producing a gain in loci and deletion events causing a loss of loci (Flajnik et al., 2001; Kelley et al., 2005). As diverse as they are, some elements of the MHC appear to be conserved. The general function and structure of the peptide presenting receptors remains consistent between species. Even the genomic organization of the 3 classes appears to be conserved in multiple species, with the exception of teleost fish (Bingulac-Popovic, Figueroa, & Sato, 1997; Kelley et al., 2005; Kuroda, Figueroa, O'hUigin, & Klein, 2002; Sato et al., 2000), where class I and II are not linked but reside on different chromosomes. For this reason the complex portion of major histocompatibility complex is dropped when referring to bony fishes and they are simply termed major histocompatibility (MH) genes (Dixon & Stet, 2001).

1.4 MH Genes and Disease

Since MH receptors present peptides from pathogens to the immune system, they are thought to play a key role in resistance/susceptibility to infectious disease. Certain alleles might encode receptors that are able to bind pathogen peptides better than receptors encoded

by other alleles. As a result, specific alleles might confer higher susceptibility or resistance to a specific disease. Research on the involvement of MH gene polymorphism in resistance or susceptibility to infectious disease in fish was limited mainly to Atlantic salmon until 2008, when researchers investigated *Salvelinus fontinalis*. The genotypes of individuals were used in conjunction with their survival status to calculate hazard ratios and percent mortalities.

For Atlantic salmon challenged with infectious salmon anemia virus (ISAv), the group of fish with the DAA*0501 allele had a lower percent mortality than the average and was thought to be associated with resistance to ISAv. On the other hand, the alleles DAA*0601, DAA*0401 and DAA*0101 had notably increased hazard ratios and were associated with susceptibility to ISAv. In a combined allele analysis for the ISAv challenge, three class II genotypes had significantly reduced hazard ratios and were associated with resistance to ISA (Grimholt et al., 2003). For the second challenge, fish were infected with *Aeromonas salmonicida*, the causative agent of furunculosis. Three class IIα alleles had significantly increased hazard ratios, meanwhile three class I alleles had significantly decreased HR's. The class II alleles DAA*0501, DAA*0701 and DAA*0101 were associated with susceptibility to furunculosis.

In a similarly structured study, brook charr were infected with *Aeromonas salmonicida*, the causative agent of furunculosis. Similar to the previous study, genotype information and survivorship was used to determine hazard ratios. In the single allele analysis, DAB*0101 had a significantly reduced hazard ratio. In contrast, the DAB*0201 allele had an increased hazard ratio although it was not statistically significant (Croisetière, Tarte, Bernatchez, & Belhumeur, 2008). The association of the DAB*0101 allele with resistance to furunculosis was also supported by the combined allele analysis. The

DAB*0101 homozygous genotype had a reduced hazard ratio and percent mortality that was not statistically significant. Conversely, two genotypes had significantly increased hazard ratios. The genotypes DAB*0301/*0401 and DAB*0201/*0301 had increased percent mortalities and were associated with susceptibility to furunculosis.

Overall, both research studies observed an association of specific alleles with better and worse performance against the respective infectious agent. This information increases the understanding of immunity in fish and can potentially be used in the aquaculture industry to breed fish with lower chances of succumbing to disease.

1.5 Purpose of the Study

The purpose of this research is to investigate the potential role of MH genes in resistance to BCWD. Previous work has observed the involvement of specific MH alleles in resistance and susceptibility to disease in brook charr and Atlantic salmon. More specifically, this work hypothesizes that a relationship exists between the MH class IIβ alleles of an individual fish and susceptibility or resistance to BCWD. Class IIβ is being investigated because it deals with extracellular pathogens like the bacteria that causes BCWD. Furthermore, if alleles involved in resistance are identified, then selective breeding could be used to produce fish stocks that are less likely to succumb to BCWD and reduce the monetary losses associated with the disease.

Chapter 2

Materials and Methods

2.1 Disease Trial and Experimental Infection of Fish

2.1.1 Fish and Rearing Conditions

Full sibling families of *Oncorhynchus mykiss* supplied from Lyndon Hatcheries Inc. were transported to the Hagan aqua lab at the University of Guelph. A maximum of six families were moved and maintained until the completion of the experimental infection trial. Fish were maintained in 60 L tanks at 11ºC and fed 1% body weight per day for three weeks to acclimate them to experimental conditions. A photoperiod of 12h light/dark was used for this experiment. Tanks were supplied with single pass well water at a flow rate of 3 L/min. Fish were divided into four separate tanks, three tanks for infection and one for a control per family.

2.1.2 Bacterial Culture Preparation and Challenge

An Ontario isolate of *Flavobacterium psychrophilum* strain 101, obtained from Lyndon Hatcheries Inc., was used for all experimental infections. The isolate was grown in cytophaga broth to an optical density of 0.6 at 600 nm (equating to 1 x 10^9 CFU/ml). This solution was adjusted to a concentration of 3.3×10^5/0.1 mL and used to infect sedated rainbow trout by intraperitoneal injection (i.p.) of 0.1 ml. After injection fish were recovered in fresh aerated water and returned to their appropriate tanks. As a negative control, one tank for each family of fish was sham-infected with 0.1 ml of sterile cytophaga broth. All procedures were performed with the approval of the University of Guelph Animal Care

Committee.

2.1.3 Sampling

Tanks were observed three times daily and any mortalities were collected and saved at -20°C. Additionally, fish showing signs of the disease such as severe lesions, hemorrhages or skin ulcerations were removed and euthanized by overdose with benzocaine at 1 g/L. After the predetermined trial period of 14 days, any surviving fish were euthanized with an overdose of benzocaine (Sigma Aldrich). Fish that survived until day 14 were recorded as survivors. In all cases, the caudal fins of both mortalities (6 per family) and survivors (12 per family) were collected, submerged in RNAlater and stored at -20ºC for later use in DNA extraction.

2.2 Cloning MH Class IIβ Sequences

2.2.1 DNA Extraction

DNA was extracted from a 0.5 cm by 0.5 cm section of the caudal fin which was initially soaked in 1.5 mL of TE buffer (100 mM Tris, 1 mM EDTA) at 4ºC for approximately 5 hours. Tissue was removed from TE buffer and transferred to tubes containing 197 µl of fish extraction buffer (100 mM Tris, 10 mM EDTA, 240 mM NaCl, 1% SDS) with 3 µl of proteinase K (300 µg/mL) and incubated overnight at 45ºC. After incubation, samples were treated with two phenol extractions (pH 6.6) and one chloroform extraction. Chilled 2-propanol was added to the final extraction product and samples were stored at -20ºC overnight to encourage DNA precipitation. Samples were centrifuged at 15,000 rpm for 15 minutes to pellet the DNA. Supernatant was removed and pellets were resuspended in TE before the addition of sodium acetate (3M acetic acid, 3 M sodium

acetate) and 95% ethanol. Samples were placed at -20ºC for 30 minutes before another centrifugation. Following the final centrifugation, supernatant was removed and DNA pellets resuspended in 50 µl of milliQ water. The final concentration of DNA was determined using a Take3 plate reader (BioTek) before samples were moved to the -80ºC for storage.

2.2.2 Primer Design and PCR

Primers have been previously designed to amplify the MH class IIβ region (Figure 2.1). The forward primer, OMYF (5'-TGCCAATTGCCTTCTACATTTGCCTG-3') was designed to the conserved region at the beginning of exon 1 (leader peptide). Meanwhile the reverse primer, OMYR (5'TGGGGGCTCAACTGTCTTGTCCAGT-3') was designed to the conserved region at the end of exon 2 (β_1 domain). PCR was carried out in a total volume of 50 µl containing 150 ng of genomic DNA, 1 mM dNTP's, 5 ul of 10X PCR buffer (10 mM Tris-HCl pH 9.0, 50 mM KCL, 0.1% Triton, 0.2 mg/mL BSA), 3 mM $MgCl_2$, 0.8 µM of both OMYF and OMYR primers, and 3 units of Taq polymerase. The fragment was amplified with the following conditions: denaturation at 95ºC for 2 minutes, followed by 24 cycles of denaturation at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 1.5 minutes. The final extension at 72ºC was carried out for 8 minutes. Two PCR reactions were performed per DNA sample.

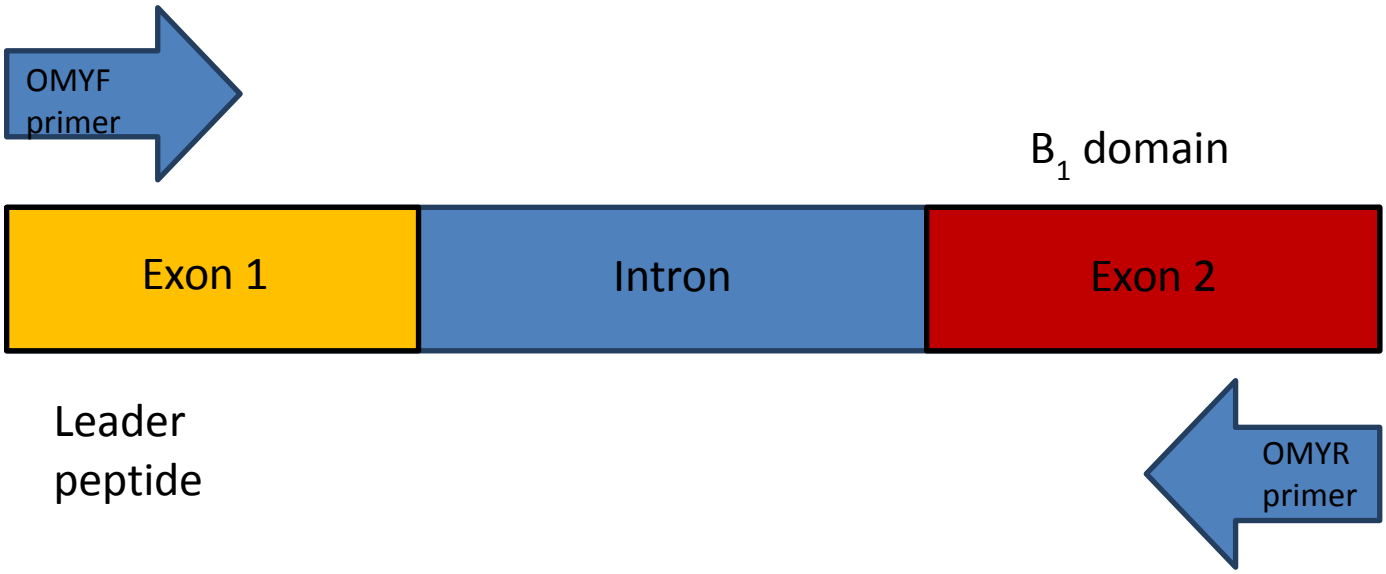

Figure 2.1 Schematic diagram of MH class II β region PCR amplified.

2.2.3 Agarose Gel Extraction

Following PCR, 8 µl of 6X loading dye (Thermo Scientific) was added to each 50 µl PCR product. The entire 58 µl reaction was loaded into a 1% agarose gel containing 3% Gel Red (Biotium) and run for approximately 40 minutes at 80 volts. The PCR product was visualized using an ultraviolet light and bands at the expected size of 750 bp were excised directly from the gel. The amplified fragment was extracted using a Qiaquick Gel Extraction Kit (Qiagen) and resuspended in 10 µl of milliQ water.

2.2.4 Ligation and Transformation

Ligation reactions were performed with 3 µl of the purified PCR product (approximately 5 ng/µl), 5 µl of 2X ligation buffer, 1 µl of pGEM-T Easy Vector and 1 µl of T4 DNA ligase. Reactions incubated at room temperature for 1 hour before 50 µl of XL1-

Blue MRF chemically competent *E. coli* cells were added. Samples were then incubated on ice for 20 minutes, heat shocked for 45 seconds in a 42ºC water bath and returned to ice for 2-3 minutes before 350 µl of SOC media was added. Cells were then allowed to recover and grow by incubation for 1 hour at 37ºC with shaking at 200-225 rpm. Following incubation, 200 µl of the transformed cells were plated on LB agar (1% tryptone, 0.5% Bacto yeast agar, 1.85 M NaCl) supplemented with 100 µg/mL of ampicillin, 20 µl of 4% 5-bromo-4-chloro-indolyl-β-D-galactopyranoside (Fermentas) and 25 µl of 0.1 M Isopropyl β-D-1-thiogalactopyranoside (Fermentas). Plates were then incubated overnight at 37ºC.

2.2.5 Plasmid Isolation

Single, well isolated white colonies were selected from the LB ampicillin plates and inoculated into 3 mL of LB ampicillin (100 µg/mL) broth. Ten colonies were picked from duplicate plates. Tubes were incubated at 37ºC overnight with shaking at 200-250rpm. The next day, culture was transferred to 1.5 mL tubes and centrifuged at 10,000 rpm for 5 minutes. The supernatant was removed and the pellets resuspended in 0.1 mL of solution I (25 mM Tris-HCL pH 8.0, 10 mM EDTA and 50 mM glucose) by vortexing. Then 0.2 mL of solution II was added (0.2 M NaOH and 1% SDS) and tubes inverted eight times and placed on ice for 5 minutes. Next, 0.15 mL of solution III (3 M potassium acetate and 11.5% acetic acid) was added and tubes inverted eight times before returning them to ice for 5 minutes. Tubes were then centrifuged at 15,000 rpm for five minutes and the supernatant transferred to a new 1.5 mL tube. The tubes containing supernatant received 0.02 mL of RNase A (1 mg/mL) and were incubated at 37ºC for 30 minutes. Following incubation, 0.3mL of cold 2-propanol was added and tubes inverted 15 times before being placed in -20ºC

overnight. The following day, tubes were centrifuged at 15,000 rpm for 15 minutes to pellet the DNA. The pellet was washed twice with 70% ethanol and resuspended in 10 µl of milliQ water. Tubes were heated at 70ºC for 10 minutes. Finally, plasmid samples were quantified with the Take3 plate reader and dilute to 1000 ng/µl. Plasmid solutions were moved to -20ºC for storage until later use.

2.2.6 Plasmid Digestion

All plasmid samples were digested to determine if the 750 bp MH class II β sequence was successfully ligated into the vector. Digestion reactions contained 1 µl of plasmid solution (1000 ng/µl), 1 µl of EcoRI (Fermentas), 2 µl of 10X Fast Digest Green Buffer and 16 µl of milliQ water. Digests were incubated at 37ºC for 25 minutes. Following digestion, 5 µl of the product was directly loaded onto a 1% agarose gel to determine the size of the insert. Samples containing an insert around 750 bp were placed in -20ºC for storage until later use.

2.2.7 Sequencing

Plasmids selected for sequencing were diluted to 200 ng/µl. Then 1 µl of plasmid solution was added to 6 µl of milliQ water before the sample was sent to The Centre for Applied Genomics, Toronto using the primers SP6 and T7. Nucleotide sequences were run through the BLAST program on NCBI to determine the identity. From each PCR product, 5 clones were sent for sequencing in both the forward and reverse direction. Alleles that were present in both of the duplicate PCR reactions were recorded in the genotype for that fish.

2.2.8 Data Analysis

Statistical analysis was performed using SPSS software (IMB). Hazard ratios were calculated by applying logistical regression and Cox regression with an event being defined as death within 14 days of experimental infection. Alleles and genotypes with increased hazard ratios ($HR>1.0$) were considered to confer susceptibility to infection and be statistically significant at $p<0.05$. Similarly, alleles and genotypes with decreased hazard ratios ($HR<1.0$) were thought to confer resistance to infection when $p<0.05$.

Chapter 3

Results

3.1 Identification of MH Class IIβ alleles from six full sibling *Oncorhynchus mykiss* families

For each individual fish, ten sequences were obtained. The *Oncorhynchus mykiss* sequences were subjected to a BLAST search using the NCBI database. The BLAST searches produced a 99% identity to known *Oncorhynchus mykiss* MH class IIβ chain alleles. A sequence alignment of all the MH class IIβ alleles observed and the longest exon two sequence in GenBank was created with the default settings on Geneious software (Biomatters). The alignment of the five alleles observed with highlighted nucleotide differences is shown in Figure 3.1.

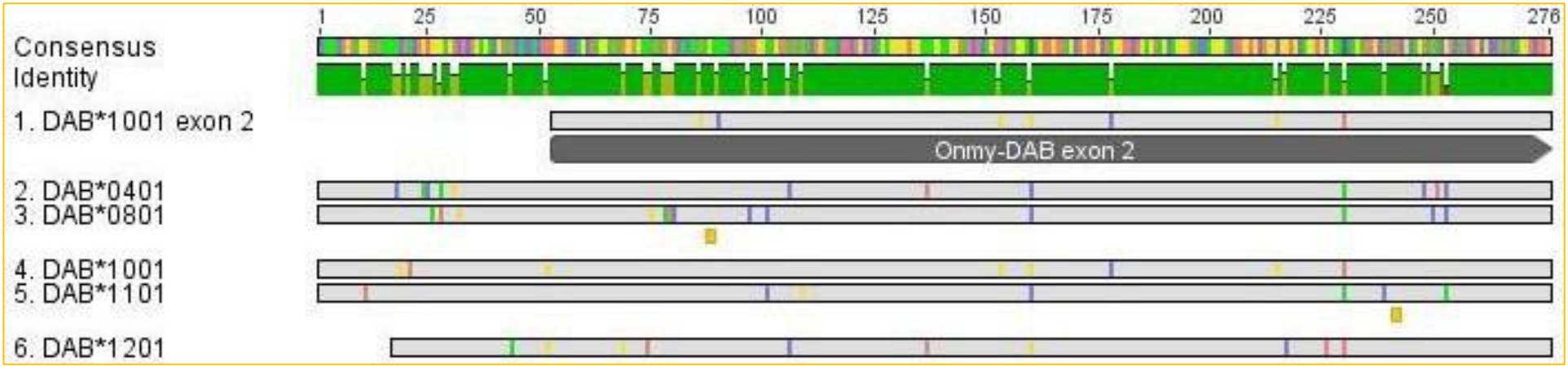

Figure 3.1 Alignment of *Oncorhynchus mykiss* MH class IIβ exon 2 nucleotide sequences. Solid grey boxes represent the nucleotide sequences and colored lines within them indicate a nucleotide difference from the consensus sequence. The DAB*1001 exon 2 region is from GenBank (Accession number: KF528072) and represents the longest partial sequence of that allele in the database. Sequences obtained from this research included introns and were trimmed to show the complete exon 2 region.

Nucleotide sequences for the complete exon two region were translated to their respective amino acids sequences using Geneious software. Amino acid sequences were subjected to a protein BLAST search using the NCBI database. The BLAST search results produced 98% identity and 100% coverage to *Oncorhynchus mykiss* MH class IIβ chain sequences (CBX11176.1, AAD53030.1, AAD53029.1, AAG02538.1, AAG02540.1). An amino acid sequence alignment of the complete exon two for all MH class IIβ alleles was created with the default settings on Geneious software. The annotated alignment of the five sequences is shown in Figure 3.2.

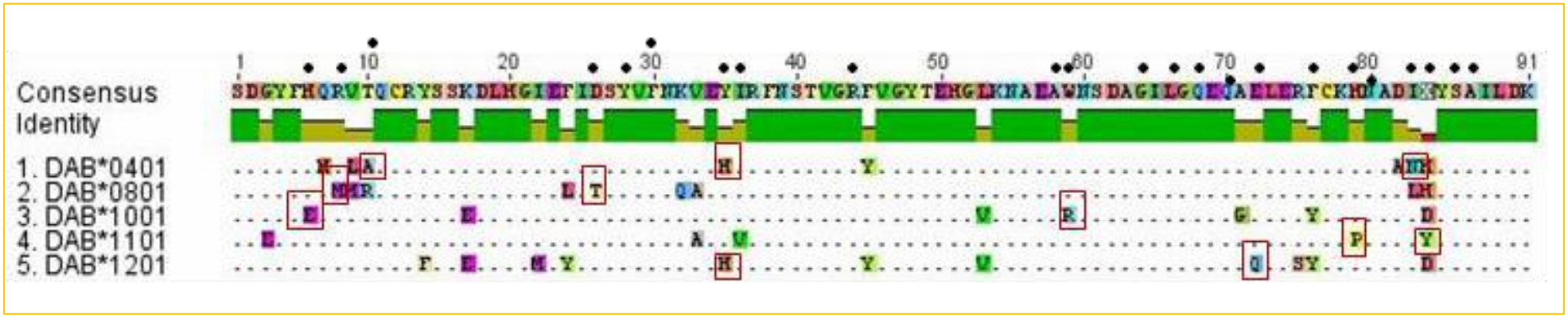

Figure 3.2 Alignment of *Oncorhynchus mykiss* MH class IIβ exon 2 amino acid sequences deduced from nucleotide sequences. Dots above the consensus sequence represent residues known to interact with the peptide in human class II receptor. Red boxes indicate unique peptide binding amino acid residues with different physical properties.

The genotype of all fish examined was recorded in an Microsoft Excel database such that fish from the same family were grouped together. Diversity and allele abundance within families was calculated. The number of times each allele was observed in a specific family was recorded and then divided by the number of individuals sequenced from that family to obtain the proportion of individuals with that allele. The occurrence of each allele was observed for every family and the results are shown in Table 3.1. Two alleles were observed to be family specific. DAB*0401 was only present in family nine, whereas DAB*1201 was only seen in family three. DAB*1001 had the most frequent occurrence, while DAB*0401 had the lowest occurrence.

Table 3.1 MH Class IIβ allele occurrence among six families of *Oncoryhnchus mykiss.* A total of 19 fish were sequenced for families four and nine, while 18 fish were sequenced for the other four families. The numbers shown are the proportion of individuals containing that allele. For example, a value of 0.56 represents 56% of the sampled fish containing that allele.

	Family Number					
	3	**4**	**6**	**7**	**9**	**10**
Allele						
DAB*401	-	-	-	-	0.21	-
DAB*801	0.56	0.11	0.61	0.33	0.47	0.50
DAB*1001	0.56	1.00	0.94	1.00	0.89	0.89
DAB*1101	0.56	0.16	0.06	-	0.16	0.50
DAB*1201	0.50	-	-	-	-	-

The genotype frequencies among the rainbow trout families were also calculated. The number of times each genotype was seen in a specific family was recorded. This number was then divided by the number of individuals sequenced from that family to obtain genotype frequency values. The recorded genotype frequencies are shown in Table 3.2. Solid dash marks indicate that the genotype was not observed. Three genotypes appeared to be family specific: DAB*1201/DAB*1001, DAB*1201/DAB*0801 and DAB*0401/DAB*1001. DAB*1201/DAB*1001 and DAB*1201/DAB*0801 were only found in family three, whereas DAB*0401/DAB*1001 was only observed in family nine. DAB*1001/DAB*1001

demonstrated the highest frequency and was observed in four of the six families. In addition to the two allele genotypes, individuals containing three and four MH class IIβ alleles were observed.

Table 3.2 MH Class IIβ genotype frequencies among the six families of *Oncorhynchus mykiss.* A total of 19 fish were sequenced for families four and nine, meanwhile 18 fish were sequenced for the other four families. Frequency was calculated using the formula f=x/n, where x is the number of times the genotype was observed and n is the number of individuals.

	Families					
	3	**4**	**6**	**7**	**9**	**10**
MH Class IIβ Genotype						
DAB*1001/DAB*1001	-	0.74	0.39	0.67	0.26	-
DAB*1001/DAB*1101	0.22	0.16	-	-	0.11	0.44
DAB*0801/DAB*0801	-	-	-	-	0.11	0.06
DAB*1001/DAB*0801	-	0.05	0.56	0.33	0.21	0.39
DAB*1101/DAB*0801	0.22	-	0.06	-	-	-
DAB*1201/DAB*1001	0.11	-	-	-	-	-
DAB*1201/DAB*0801	0.28	-	-	-	-	-
DAB*0401/DAB*1001	-	-	-	-	0.05	-
DAB*0801/1001/1101	0.11	0.05	-	-	0.05	0.11
DAB*0401/0801/1001	-	-	-	-	0.16	-
DAB*1001/1101/1201	0.06	-	-	-	-	-
DAB*0401/0801/1001/1101	-	-	-	-	0.05	-

As seen in Table 3.2, genotypes with more than two alleles were observed. The genotype data base was used to determine which and how many families contained individuals with more than two MH class IIβ alleles. Families that possessed individuals with two, three and four alleles respectively are shown in Table 3.3. Families are represented in the columns of the table, while the number of alleles are represented in the rows. Check marks indicate cases where that number of alleles/individual were found, whereas dash marks designate where they were not observed. Individuals with four alleles were only observed in family nine, whereas individuals with three alleles were only seen in families three, four, nine and ten.

Table 3.3 Number of MH class IIβ alleles found per individual in *Oncoryhnchus mykiss* families.

	Family Number					
	3	**4**	**6**	**7**	**9**	**10**
Alleles/Individual						
Two	✓	✓	✓	✓	✓	✓
Three	✓	✓	-	-	✓	✓
Four	-	-	-	-	✓	-

3.2 Identification of MH Class IIβ alleles in survivors and mortalities

The excel database was reorganized to group fish containing the same allele or genotype and then further subdivided into a group of mortalities and survivors. This allowed for the observation of single allele and genotype prevalence in survivors and mortalities. The prevalence of each allele in mortalities and survivors is shown in Table 3.4. Due to the bacterial challenge results, there were a higher number of mortalities examined. DAB*1001 was observed in the highest percentage in mortalities and survivors. Meanwhile DAB*0401 was seen in the lowest percentage of both groups. Similarly, the results of genotype prevalence are shown in Table 3.5. Both DAB*1001/DAB*1001 and DAB*1001/DAB*0801 were observed at the highest frequency in survivors and mortalities. However, both genotypes were seen in a higher frequency of survivors. Three allele genotypes occurred in both survivors and moralities at a very low frequency. In contrast, the four allele genotype was only observed in the survivor group at an extremely low frequency.

Table 3.4 Single allele counts in mortalities and survivors. Alleles are represented in the table rows and the number of fish with that allele in each of the two groups are represented in the columns. A G-Test was performed on this contingency table using SPSS statistical software and the result is shown below.

Alleles	Number of Mortalities	Number of Survivors
DAB*0801	27	20
DAB*1001	53	45
DAB*1101	18	11
DAB*1201	6	3
DAB*0401	3	1

G = 16.094

p-value = 0.446

Table 3.5 Genotype counts in mortalities and survivors. A G-Test was performed on this contingency table using SPSS statistical software and the result is shown below.

Genotype	Number of Mortalities	Number of Survivors
DAB*1001/DAB*1001	20	19
DAB*1001/DAB*1101	11	6
DAB*0801/DAB*0801	2	1
DAB*1001/DAB*0801	12	15
DAB*1101/DAB*0801	4	1
DAB*1201/DAB*1001	2	1
DAB*1201/DAB*0801	3	1
DAB*1201/DAB*1101	0	0
DAB*0401/DAB*1101	0	0
DAB*0401/DAB*1001	1	0
DAB*0401/DAB*0801	1	0

G = 44.436

p-value = 0.993

3.3 Identification of MH Class IIβ alleles conferring resistance or susceptibility to Bacterial Cold Water Disease

The excel database in its reorganized format, grouping individuals by allele and genotype, was used to perform several survivor analyzes. A Cox regression was performed on single MH class IIβ allele data with default settings using SPSS statistical software. Cox regression utilizes allele information, survivor status and time until death to calculate hazard ratios. The resulting hazard ratios are shown in Table 3.6 with their corresponding p-values. Hazard ratios are considered statistically significant when $p < 0.05$. DAB*0401 had the highest hazard ratio, while DAB*1001 had the lowest.

Additionally, SPSS software was used to carry out a logistic regression to calculate hazard ratios for individual alleles. In contrast to the Cox regression, the logistic regression only uses allele information and survivor status to calculate hazard ratios. The resulting hazard ratios for the logistic regression are shown in Table 3.7 with their corresponding p-values. Ratios are statistically significant if $p < 0.05$. DAB*0401 had the highest hazard ratio, while DAB*1001 and DAB*0801 had the lowest hazard ratios, but none of the calculated hazard ratios were significant.

Table 3.6 Calculated Hazard Ratios for single MH Class IIβ alleles using Cox Regression.

Allele	Hazard Ratio	p-value
DAB*0401	1.669	0.394
DAB*0801	0.951	0.868
DAB*1001	0.635	0.319
DAB*1101	1.243	0.461
DAB*1201	1.018	0.696

Table 3.7 Calculated Hazard Ratios for MH Class IIβ alleles using Logistic Regression.

Allele	Hazard Ratio	p-value
DAB*0401	2.728	0.395
DAB*0801	0.872	0.753
DAB*1001	0.368	0.232
DAB*1101	1.318	0.549
DAB*1201	1.113	0.898

Similar to the single allele analysis, a Cox regression was performed on MH class IIβ genotypes using SPSS statistical software. The calculated hazard ratios are shown in Table 3.8 with their respective p-values. DAB*0401/DAB*1001 and DAB*1101/DAB*0801 had the highest hazard ratios, while DAB*1001/DAB*1001 and DAB*1001/DAB*0801 had the lowest. Additionally, a logistic regression was performed on the same MH class IIβ genotypes using SPSS software. Hazard ratios are shown in table 3.9 with their calculated p-values, although none were significant. Three genotypes tied for the highest hazard ratio, while DAB*1001/DAB*0801 had the lowest observed hazard ratio.

Table 3.8 Calculated Hazard Ratios for MH Class IIβ genotypes using Cox Regression.

Allele	Hazard Ratio	p-value
DAB*1001/DAB*1001	0.835	0.699
DAB*1001/DAB*1101	1.471	0.447
DAB*0801/DAB*0801	1.421	0.667
DAB*1001/DAB*0801	0.790	0.633
DAB*1101/DAB*0801	2.170	0.202
DAB*1201/DAB*1001	0.917	0.916
DAB*1201/DAB*0801	1.383	0.691
DAB*0401/DAB*1001	4.465	0.171

Table 3.9 Calculated Hazard Ratios for MH Class IIβ alleles using Logistic Regression.

Allele	Hazard Ratio	p-value
DAB*1001/DAB*1001	0.877	0.848
DAB*1001/DAB*1101	1.528	0.592
DAB*0801/DAB*0801	1.667	0.708
DAB*1001/DAB*0801	0.722	0.649
DAB*1201/DAB*1001	1.667	0.708
DAB*1201/DAB*0801	1.667	0.708
DAB*0401/DAB*0801	1.20	0.763

Chapter 4

Discussion

4.1 Overview

The work performed in this thesis represent the first time Lyndon Hatchery Inc. rainbow trout families have been genotyped for MH genes. Additionally, it is the first investigation into survival conferred by specific MH class IIβ alleles and genotypes in a salmonid species other than Atlantic salmon or brook charr. A total of 18 fish from six families of rainbow trout infected with *Flavobacterium psychrophilum* were sampled and genotyped for MH class IIβ. This resulted in a genetic profile for each family and insight towards family diversity with respect to MH genes. Furthermore, allele and genotype information was used in a survival analysis by performing a Cox regression and binary logistic regression to examine their involvement in resistance or susceptibility to BCWD in disease trials.

4.2 Examining the MH Class II β_1 domain structure

For all alleles identified, the exon 2 region (β_1 domain encoding) exhibited more nucleotide variability than either the exon 1 (leader peptide encoding) or intron region. This variability can be seen at the protein level where many amino acid differences were observed in the translated exon 2 alignment (Figure 3.2). These findings are consistent with the observations of variability in the human class II gene and receptor (H. J. Brown et al., 1993; Gonzalez-Galarza et al., 2011). Additionally, similar variability has been found in the nucleotide and amino acid sequences of MH class IIβ for other salmonid species (P

Conejeros, Power, Alekseyev, & Dixon, 2012; Croisetière, Tarte, Bernatchez, & Belhumeur, 2008; Dixon, Nagelkerke, Sibbing, Egberts, & Stet, 1996; Noakes, Reimer, & Phillips, 2003).

Amino acid residues of the β_1 domain that directly interact with the antigen have been identified for the human class II receptor but remain to be elucidated for salmonids (H. J. Brown et al., 1993; Gonzalez-Galarza et al., 2011). Therefore positions from the human receptor are often used as a reference to indicate potential antigen interaction sites. Out of the five alleles obtained, DAB*0801 had the most amount of unique residues. There are seven unique residues, five with different physical properties than the consensus sequence and two of which exist at suspected antigen interaction sites. At position eight the amino acid residue differs from a positively charged arginine to a hydrophobic sulfur containing methionine. Meanwhile the amino acid residue is changed from a hydrophilic threonine to a positively charged arginine at position 10: and a negatively charged aspartic acid or hydrophilic tyrosine to a positively charged histidine at position 84. However the amino acid change at position 84 is not unique because it is shared by DAB*0401.

Both DAB*1201 and DAB*0401 have the second highest number of unique residues. Each possesses five unique residues, all with different physical properties. For DAB*0401, two of these exist at suspected antigen contact sites, whereas only one is present at an interaction site for DAB*1201. In DAB*0401 the amino acid is a hydrophilic threonine opposed to a hydrophobic alanine at position 10 and a hydrophobic isoleucine rather than a negatively charged glutamic acid at position 83 respectively. In DAB*1201, the residue is a hydrophobic glutamic acid opposed to a hydrophilic glutamine.

The last two alleles, DAB*1101 and DAB*1001 contain four and three unique residues respectively. For DAB*1101, three residues have different physical properties and two of them exist at possible antigen interaction positions. At position 79 the amino acid residue is a positively charged histidine opposed to a hydrophobic proline. At position 80, the residue is a negatively aspartic acid or a positively charged histidine opposed to a hydrophilic tyrosine. For DAB*1001, only two of the residues with different physical properties are at the suspected antigen interaction sites. At position six a positively charged histidine exists, opposed to a negatively charged glutamic acid. Meanwhile at position 84 the amino acid is a positively charged histidine or hydrophobic tyrosine opposed to a negatively charged aspartic acid. Note that this is not a unique residue change because it is shared with DAB*1201.

It is important to consider amino acid variability in the β_1 region as it has a direct influence on peptide binding properties. The functional aspect of this variability and its role in resistance or susceptibility to disease has only been studied in a handful of fish species (Croisetière et al., 2008; Grimholt et al., 2003; Langefors, Lohm, Grahn, Andersen, & von Schantz, 2001). Previous research has shown that a single amino acid change correlated with increased resistance to disease (Langefors et al., 2001). The study by Langefors and colleagues identified a histidine at position ten in the β_1 domain that was responsible for resistance to furunculosis in European populations of Atlantic salmon. Similarly, in work by Croisetière and colleagues, researchers identified a tyrosine at position nine that was associated with resistance to furunculosis in brook charr and exclusive to the DAB*0101 allele. In both cases researchers stated that the presence of those specific residues could be critical for efficient presentation of *A. salmoncidia* antigens. The presence of critical residues

for antigen presentation could also be the case with rainbow trout and *F. psychrophilum*. Unique residues with altered physical properties were observed for specific alleles. Some of these residues might bind to *F. psychrophilum* proteins better and lead to more efficient antigen presentation and a stonger adapative immune response.

Researchers have examined several *F. psychrophilum* proteins in attempt to identify antigenic targets for vaccine use (Crump, Burian, Allen, & Kay, 2005; F Dumetz, Lapatra, Duchaud, Claverol, & Le Hénaff, 2007; Fabien Dumetz et al., 2008; Massias, Dumetz, Urdaci, & Le Hénaff, 2004). Proteins identified to have antigenic properties range from hydrophobic, polar and weakly charged peptides, to nonpolar or highly charged peptides (Crump et al., 2005; F Dumetz et al., 2007; Massias et al., 2004). With such variability in the properties of these peptides it is difficult to hypothesize which MH receptor is better suited to bind *F. psychrophilum* proteins. Knowing what alleles encode for a more hydrophobic or hydrophilic peptide binding groove will be more useful in protein-protein interaction studies that examine specific antigenic peptides and MH groove interaction.

4.3 Genetic Profiles of six Lyndon Hatchery Families

From the six families of rainbow trout a total of five alleles were identified. This is much fewer than what has previously been observed by the lab and other researchers (Conejeros, Power, Alekseyev, & Dixon, 2012; Conejeros et al., 2008; Dorschner, Duris, Bronte, Burnham Curtis, & Phillips, 2000; Grimholt et al., 2003). Conejeros and colleagues found 40 different MH class IIβ alleles in 55 Arctic charr sampled from various locations across the globe. Similarly, Dorschner and colleagues looked at MH class II in 74 lake trout from Lake Superior and obtained 43 different alleles. In contrast, research by Grimholt and

colleagues on Atlantic salmon obtained seven MH class IIα and IIβ alleles from 82 fish. There are two possible explanations for this low allele diversity. These families are cultured rainbow trout that have likely been bred and selected for faster growth, better feed to weight conversion and better flesh quality. There has been no specific selection with regard to MH genes and therefore the low diversity might simply be a by-product of selection for other desirable traits. Instances where a high number of alleles have been observed previously were obtained from wild populations of fish. In these populations, humans do not have as much influence on breeding and a higher level of allele diversity might be maintained naturally. However this might not always be the case. Research by Croisetière and colleagues found six MH class IIβ alleles from 23 wild brook charr families. Wild charr were used to generate full and half sib families for the study with no additional generational selection being apparent. The second potential reason for the low number of alleles observed in this thesis is that only six families out of the 60 families from Lyndon Hatcheries were genotyped. By chance it could be that these families just happen to be more similar with regard to MH genes than the other families, but since the other families were not genotyped it is not possible to know for certain.

Of all the families, nine and three displayed higher allele diversity with each group possessing four of the five class II alleles. In family three, each of the alleles was found in approximately 50% of the individuals. In contrast, family nine showed differences in allele frequency. The allele DAB*1001 was observed in a higher number of individuals than the other three alleles seen in families three and nine. It was seen in 89% of individuals, whereas DAB*1101 was only found in 16% of individuals. Both families nine and three had mortality

rates of 65% and 53.1% respectively. These are moderate moralities rates in comparison to other families during the same experimental infection trials. Families four, six and ten have the same three alleles, but not in the same frequencies. Only DAB*0801, DAB*1001 and DAB*1101 were observed in these families. DAB*1001 was observed in over 89% of individuals in all three families. DAB*0801 was seen in 50% of individuals in family ten, 61% of individuals in family six and only 11% of individuals in family four. Similar to DAB*0801, allele DAB*1101 was observed in 50% of family ten. However the allele DAB*1101 was only found in 16% and 6% of individuals from families four and six, the two families which demonstrated mortality rates of 30% and 39% respectively. Families four and six demonstrated lower mortality rates, while family ten had the highest mortality rates. The last family examined, number seven, demonstrated the lowest mortality rates and also showed the lowest allelic diversity. In this family only DAB*0801 and DAB*1001 were observed. DAB*1001 was found in all individuals, whereas DAB*0801 was observed in 33%. It is interesting to see that just two alleles exist in the most resistant family, but these alleles also exist in all of the other families. Additionally both alleles were observed at a similar frequency in other families. Furthermore, a higher level of homozygous individuals was observed in families four and seven. Lastly, of all the alleles observed just two were exclusive to a single family. DAB*0401 was only seen in family nine and DAB*1201 was solely found in family three. With this information alone, it is difficult to speculate what the family differences could represent. Therefore to get a better image of family genetic profiles, genotype frequencies were calculated.

There were several interesting observations from the table of calculated genotype frequencies. A total of eight different "two allele" genotypes were found among the rainbow

trout families. The genotype DAB*1001/*0801 was observed in five of the families and had the highest frequency in family six. This was one of the more resistant families and it is interesting to note that the genotype is a combination of the sole two alleles found in the most resistant family. DAB*1001/*0801 also existed in family seven but at a slightly lower frequency. This observation suggests that having a high level of the genotype DAB*1001/*0801 in the population might produce higher levels of resistance. The genotype DAB*1001/*1001 was seen in four of the families. This genotype had the highest frequency in the most resistant families, four and seven. It is noteworthy that this is one of only two homozygous genotypes and the other genotype DAB*0801/*0801 was only observed in two families in very low frequency.

Similar to the previously mentioned genotypes, DAB*1001/*1101 was found in four of the six families. But in contrast to DAB*1001/*1001 and DAB*1001/*0801 it was observed at the highest frequency in family ten and not at all in family seven. Its presence in the most susceptible family and absence in the most resistant is particularly interesting. Even though it exists in other families at lower frequencies genotype DAB*1001/*1101 may still have a role in susceptibility. In the genotype examination, DAB*1201 was only found in three genotypes, with DAB*1001, DAB*0801 and DAB*1101 and at low frequency. Similarly, DAB*0401 was exclusive to family nine and only found in a two allele genotype with DAB*1001 and at low frequency.

The last notable observation is the presence of some fish with three and four MH class II alleles. The occurrence of two or more alleles per individual has been commonly observed in previous MH studies (Conejeros et al., 2012; Conejeros et al., 2008; Dixon,

Nagelkerke, Sibbing, Egberts, & Stet, 1996; Dorschner et al., 2000; Kruiswijk et al., 2004; Noakes, Reimer, & Phillips, 2003) but is usually attributed to high MH polymorphism. Another proposed explanation for this observation is that the alleles come from two different loci. If alleles did in fact come from duplicate loci, researchers would expect to see evidence in the phylogenetic tree as two major branches with clusters of alleles in each, however this was not the case (Dorschner et al., 2000). Instead, Dorschner and colleagues observed a subdivided tree with many small clusters of alleles.

So why do some individuals have two alleles while other have three or four? Of the 110 rainbow trout analyzed in this thesis work ten individuals were found to have three alleles and one individual was found to have four alleles. Due to the nature of MH sequences and the cloning process, PCR artifacts and false alleles can arise (Lenz & Becker, 2008). Therefore precautions were taken by modifying the PCR protocol to minimize their formation (Lenz & Becker, 2008). Hence there is high confidence that these sequences and genotypes represent real alleles. There are two possible explanations for why some individuals appear to have multiple loci while others do not. The first reason is differential amplification due to primer bias. If there are commonly two loci present, yet primers bind more strongly to one locus during PCR, preferential amplification would occur and misrepresent the sequences present. However this does not seem to be the case because the low frequency genotypes with three alleles are comprised of alleles found in the other common and high frequency genotypes. The second possible reason is that MH class IIβ genes are arranged in haplotypes that vary in their number of loci. This was demonstrated to be the case in a study of MH genes in cichlid fish (Málaga-Trillo et al., 1998) but remains to

be elucidated for salmonids. Lastly, researchers have proposed a theory to explain the varying number of MH loci (Málaga-Trillo et al., 1998). They hypothesize that species will vary in the number of functional loci they contain, and that the presence of multiple functional MH loci would reduce an individual's collection of T-cells due to elimination of self-reactive cells. It is thought that this could impair ones ability to combat pathogens.

4.4 Resistance and Susceptibility: Analysis of MH Class IIβ Allele involvement

Out of all six families, ten had the highest mortality rate at 85% and was the most susceptible. Families nine and three had mortality rates of 65% and 53% respectively. Family six and four had the most similar mortality at 39% and 30%. Lastly, family seven had the lowest mortality at 9% and was the most resistant family (Lowia Al-Hussinee, personal communication).

Single allele prevalence in groups of mortalities and survivors was the first thing examined, in order to ascertain any potential resistance or susceptibility effects. However, all alleles were observed in both survivors and mortalities with minimal differences in prevalence between those two groups. DAB*0801, DAB*1201 and DAB*0401 had less than a 5% difference in prevalence between the two groups. Whereas DAB*1101 and DAB*1001 had differences of 6% and 9% respectively. Interestingly, DAB*1001 was the most frequently observed allele but also found to be in a higher amount of survivors, however only at 10% difference. This has to do with the high number of homozygous DAB*1001 survivors from family seven. When analyzed by the G-Test, the differences between the group of mortalities and survivors was not found to be significant.

In addition to prevalence, hazard ratios were calculated by Cox and logistic regression for each allele individually. Although none of the calculated HR's demonstrated statistical significance there are some noteworthy observations. DAB*1001 had the lowest HR in both regressions, suggesting increased resistances. In contrast, DAB*1101 and DAB*0401 had the highest HR's, suggesting increased susceptibility. Hazard ratios calculated between the two regressions were fairly similar with the exception of DAB*0401. For the Cox regression the HR was calculated to be 1.669, whereas it was 2.728 for the logistic regression. This is a result of this allele's extremely low frequency in this study. Due to the nature of the logistic regression the HR's can be greatly affected by low frequency events. When HR's are calculated with logistic regression only binary information (dead/alive) is used. Therefore the presence of one allele in low frequency and in only a couple mortalities, would produce a largely increased HR that might not accurately reflect the true hazard presented by possessing this allele. That is why it is important to consider allele frequency information in tandem with survival information. Lastly, the Cox regression uses more information than the logistic, taking into account time until death. Calculation of HR's by Cox regression was a main goal of this study and logistic regression was carried out solely on curiosity and for comparison.

4.5 Resistance and Susceptibility: Analysis of MH Class IIβ Genotype involvement

Similar to the single allele analysis, genotype information was examined first by prevalence in mortalities and survivors, and then by Cox and logistical regression. Like the single allele analysis most genotypes had minimal differences in prevalence between the group of survivors and mortalities with the exception of two genotypes. The genotypes

DAB*1001/*0801 and DAB*1001/*1001 had the largest differences between survivors and mortalities, at 12% and 8% respectively (Table 3.5). Both genotypes existed in a higher amount of survivors than mortalities. For DAB*1001 this was also observed by differences in single allele prevalence (Figure 3.4). Interestingly, these are the only two genotypes found in family seven, the most resistant family. Lastly, when analyzed by the G-Test, differences between mortalities and survivors was not statistically significant.

In the Cox and logistic regression both genotypes DAB*1001/*1001 and DAB*1001/*0801 had the lowest HR's, albeit not statistically significant. The reduced hazard ratios suggest increased resistance, further supporting the observations from the single allele analysis of DAB*1001. Therefore DAB*1001 could play a subtle role in increased resistance. In contrast, there were two genotypes that showed higher HR's than the rest, but were not statistically significant. In the Cox regression, both DAB*1101/*0801 and DAB*0401/*1001 had the highest HR's. Hazard ratios were also calculated by logistic regression but the results for these two genotypes were not included in Table 3.9 because of their misleading nature. Both genotypes had HR's well over 1000 and this was because they had extreme low frequency, which skews the HR's as seen in the single allele analysis. For this reason a HR for the genotype DAB*0401/*1101 was not shown in either the Cox or logistic regression.

The results of this study do not strongly indicate that any MH class IIβ allele or genotype has a significant role in resistance or susceptibility to BCWD in a 14 day mortality trial. This is unlike previously published work on brook charr and Atlantic salmon which found that certain MH alleles are significantly associated with disease resistance or susceptibility (Croisetière et al., 2008; Grimholt et al., 2003). There are two possible

explanations for this difference. First, the nature of the pathogen. Recall that *F. psychrophilum* is thought to be a facultative intracellular bacteria and has been observed in kidney phagocytes of rainbow trout (Madetoja, Nyman, & Wiklund, 2000; Wiklund & Dalsgaard, 2003). The amount of time the bacteria spends inside a host cell versus in extracellular spaces would dictate the effectiveness of a class I or class II response. If the pathogen spends significant time inside the host cell then a class I response would be more appropriate and might have a more important role in resistance or susceptibility. Therefore, it might be the combination of class I and class II genes that determine resistance and susceptibility to BCWD. The second possibility is that innate immunity plays a larger role than adaptive in this case, as adaptive immunity takes much longer to initiate. The 14 days of the infection trial period might not have been long enough for the adaptive immune system to generate a full response and have effects on survivorship. Instead, aspects of the innate immune system would have been in full effect and could have affected survivorship. And perhaps differences in the innate immune system of these families could have been the cause of differential survivorship. Therefore rather than selecting fish based on MH genotype, fish farmers would want to perform selection based on innate immune function.

4.6 Challenges of Examining Genetic Disease Resistance

When evaluating a specific gene for potential involvement in resistance or susceptibility to disease it is important to remember that other genes might be involved. Some aspect of the genetic background not being investigated could be responsible for the observed effect. This could result in falsely attributed resistance or susceptibility effects for alleles of the gene in question. In previously published work evaluating genetic disease

resistance in aquaculture species, controlling for genetic background was found to be difficult and no standard methods have been well established (Croisetière et al., 2008;Grimholt et al., 2003; Ødegård, Baranski, Gjerde, & Gjedrem, 2011). However, it is believed that selection of individuals from different families can aid in minimizing genetic background effects (Croisetière et al., 2008). During analysis and calculation of HR's for this work, individuals were grouped by genotype and not by family. Families are thought to have variable genetic backgrounds because they have different parents and mixing individuals from different families for the analysis should minimize effects of the genetic background. This reduces the chance of falsely attributed resistance or susceptibility.

4.7 Conclusions

This study investigated the role of MH class IIβ alleles on resistance and susceptibility to BCWD in six families of rainbow trout from Lyndon Hatcheries Inc. A low level of diversity was observed with respect to MH genes as only six alleles were found from 110 individuals. This was thought to be the byproduct of selective breeding for other desirable and unrelated market traits. Similar to other MH gene studies, high nucleotide and amino acid polymorphism was observed in the exon 2 (β_1 encoding) region. Both the allele DAB*1001 and the genotypes DAB*1001/1001 and DAB*1001/*0801 were observed in a slightly higher percentage of survivors than mortalities. In addition, they had the lowest observed hazard ratios, suggestive of an association with resistance. Although the association was not statistically significant, it shows potential and would be worth investigating further. Lyndon hatcheries has at least 30 other rainbow trout families that have not been examined for MH genotype. These families could have an equal or higher

level of genetic diversity than those families examined in this study. The other families should be genotyped for major histocompatiblity so that MH genetic diversity of their farm can be investigated. If low MH diversity is observed, then perhaps the breeding program should be reexamined.

4.8 Future Work

DNA samples from fish used in this study could be used to investigate the role of MH class I in resistance or susceptibility through an additional set of histocompatibility genotyping. This new information could be analyzed on its own and in tandem with the MH class II genotypes.

Another option for future research would be to do a repeat trial with fish from the same families, but have a lower dose of infection and have the trial period run longer. It would be interesting to see if the overall mortalities rates were similar to this trial and if the genotypic profile of survivors and mortalities varied from what was observed in this study. Lastly, if resources were available, it would be worth taking a look at aspects of the innate immune system and investigating any differences between the families.

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