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Serum IgM, MH class IIβ genotype and respiratory burst activity do not differ between rainbow trout families displaying resistance or susceptibility to the coldwater pathogen, *Flavobacterium psychrophilum*.

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Abstract

Flavobacterium psychrophilum, the causative agent of bacterial coldwater disease (BCWD) is a significant threat to global aquaculture. True to its name, BCWD tends to occur at temperatures between 8-12^{o}C and presents as a systemic disease with characteristic skin ulcerations. Juvenile rainbow trout are particularly susceptible and in these fish the condition is referred to as rainbow trout fry syndrome (RTFS). Resistance to *F. psychrophilum* is heritable and is not adversely correlated with the growth of fish, thus selective breeding appears to be an achievable approach to its control. The current study explores the connection between resistance to BCWD and several immunological markers. After determining resistance/susceptibility to *F. psychrophilum* following experimental infection in 40 full-sibling families of rainbow trout, selected families were experimentally infected with *F. psychrophilum* and differences in antibody production, major histocompatibility (MH) class IIβ genotype and respiratory burst activity (RBA) throughout infection were compared. Serum IgM production increased over time but significant differences between resistant and susceptible families were not observed at either 28 days or 120 days. Of the six families that were genotyped for MH class IIβ, there did not appear to be specific genotypes that conferred resistance or susceptibility to *F. psychrophilum*. Further, the RBA of both head kidney leukocytes and whole blood was not significantly different between the resistant and susceptible rainbow trout families. Although the selected immune markers did not differ based on resistance status, the RBA of head kidney leukocytes in all families studied dramatically decreased seven days after infection while total blood RBA remained constant. Day seven was also when severe symptoms and/or mortality due to BCWD was first observed, thus these results may reveal information regarding the pathogenesis of the organism. A better understanding of appropriate immune defenses could provide the basis for breeding programs to effectively combat this costly pathogen, but further study of functional immune markers particularly during the fry stage of development is required.

Keywords: rainbow trout, BCWD, major histocompatibility, respiratory burst, antibody

1. Introduction

A large obstacle to overcome when dealing with aquaculture production of any type is the vulnerability to disease outbreaks. Like other forms of agriculture, aquaculture places animals in a setting that deviates significantly from that of their natural environment. The stress induced by conditions such as overcrowding, temperature fluctuations and handling can result in normally benign microorganisms becoming opportunistic pathogens (Meyer, 1991). One such pathogen causing significant losses in the farming of rainbow trout (*Oncorhynchus mykiss*) is *Flavobacterium psychrophilum*, the etiological agent of bacterial cold-water disease (BCWD). Rainbow trout, a coldwater salmonid, is an important species of choice in global aquaculture, which has enabled BCWD to become a worldwide concern.

F. psychrophilum is widespread in freshwater aquatic environments but typically only causes infection in intensively reared salmonids at temperatures lower than 16^{o}C (Holt, 1987). Although *F. psychrophilum* can infect many salmonid species and some non-salmonid species, juveniles of coho salmon (*Oncorhynchus kisutch*) and rainbow trout are particularly susceptible (Nematollahi et al. 2003, Starliper, 2011). Clinical signs and manifestations vary depending on location as there appears to be geographic variability between isolates (Lumsden et al., 2004). In North America, the most common presentation of BCWD in fish larger than fingerling is an ulcerative dermatitis known as peduncle disease (Lumsden et al. 2004). The nature of this presentation means that the financial losses associated with BCWD are not due to mortalities alone as surviving fish often produce fillets that are less valuable or not marketable (Lumsden et al, 2004). In fry, the most important presentation is a systemic disease, often with exopthalmia and anemia, known as rainbow trout fry syndrome (RTFS, Bebak et al. 2007). With no commercial vaccine available and mortality, although variable, has been reported to be as high as 50-85% (Brown et al, 1997, Cipriano and Holt, 2008), the financial losses due to *F. psychrophilum* infection can be devastating.

To combat the significant losses due to infectious disease, there has been increasing interest in selectively breeding aquaculture stocks for disease resistance. As has been demonstrated with many other

aquatic diseases (Gjedrem 1991, Henryon 2005, Kjoglum 2006, Kjoglum 2008), resistance to BCWD does appear to be heritable (Silverstein et al. 2009), but there has been little research to identify the basis for this resistance. In addition, a method to consistently produce resistant crosses is yet to be developed for the vast majority of aquatic organisms. Creating and challenging families of rainbow trout takes a great deal of time and finances, thus identifying a marker for the observed resistance to BCWD would decrease costs and expedite this process immensely. With the immune system playing a vital role in the prevention and clearance of infection, it is reasonable to hypothesise that the heritable resistance to BCWD may be associated with teleostean immune function.

Like mammals, teleosts have both innate and adaptive immune systems. The innate immune response constitutes the first line of defense against infection and is activated through recognition of conserved molecular patterns common to pathogenic organisms (Uribe et al. 2011). Phagocytosis, or the engulfment of particles, is a major component of immunity in fish (Uribe et al. 2011). Circulating phagocytes will take up foreign bodies such as bacteria and eradicate them through the production of reactive oxygen species (ROS) during a respiratory burst (Nikoskelainen et al. 2004). Phagocytosis, and the killing activity associated with it, is reported to be the mechanism of innate immunity that is the least influenced by low temperatures (Callazos et al. 1994a, Callazos et al. 1994b, Nikoskelainen et al. 2004), thus this process may be critical when dealing with a cold-water pathogen such as *F. psychrophilum*.

While the innate immune system is fast-acting, adaptive immunity requires more time to develop and has greater specificity. A key component of vertebrate adaptive immunity is the generation of antigen-specific antibodies from B cells. Antibodies promote the control and clearance of infection through such processes as opsonisation for phagocytes, complement activation and neutralization of microbes and toxins (Forthal, 2014). To date, three classes of immunoglobulins have been discovered in teleosts: IgM, IgT and IgD (Sunyer, 2013). The most abundant immunoglobulin is a tetramer of the IgM class (Fillatreau et al. 2013). IgM is the first antibody class to appear after initial exposure to an antigen and may be part of an effective

humoral response against invading pathogens (Fillatreau et al. 2013, Reyes-Cerpa et al. 2013). With these protective properties in mind, there have been numerous attempts to identify antigens capable of inducing an effective antibody response to combat *F. psychrophilum* (LaFrentz et al. 2002, Crump et al. 2005, Dumetz et al. 2006, Sudheesh et al. 2007). Although antibody titres have been observed to increase after immunization with many of these antigens and whole-cell lysates, similar increases were often observed with adjuvant alone (LaFrentz et al. 2002). As there is limited information regarding consistent infection and vaccination models for *F. psychrophilum* (reviewed in Gomez et al. 2014), there is a critical need to better understand the serum IgM response in fish presenting resistance/susceptibility to BCWD.

Although fish farmers can benefit from breeding programs based solely on survival phenotype, identifying genes (markers) correlated with enhanced immune performance could aid in the more rapid development of optimum stocks. One possible marker, and a focus of this study, is the major histocompatibility (MH) class II genes. As the class of molecules that recognize and present antigens from extracellular pathogens to tailor the resulting immune responses (reviewed in Neefjes et al. 2011), there has been a great deal of interest in determining whether specific MH class II genotypes are linked to bacterial resistance/susceptibility in fish species (Xu et al. 2010, Dionne et al. 2009, Rakus et al. 2009, Kjoglum et al. 2008). Conversely, MH class I is associated with recognition and presentation of antigens from intracellular pathogens such as viruses (reviewed in Neefjes et al. 2011). Because *F. psychrophilum* appears to have both intracellular and extracellular tendencies throughout its infection cycle (Wood and Yasutake, 1956, Decostere et al. 2001, Nematollahi et al. 2005, Nilsen et al. 2011), it is not fully understood whether MH class I or MH class II genes would provide more protection towards BCWD. To date there has been some evidence of an association between MH class II variability and resistance to *F. psychrophilum* (Johnson et al. 2008), but there has been minimal research since then to relate specific alleles with BCWD resistance. As such, one component of this study aims to link MH class IIβ alleles with observed resistance to BCWD and determine whether this association goes further.

In the present study, full-sibling families of rainbow trout displaying resistance/susceptibility to BCWD were experimentally infected with *F. psychrophilum* so that differences in MH class IIβ genotype, respiratory burst activity and antibody production could be observed and compared. As there is limited knowledge regarding both the pathogenesis of *F. psychrophilum* and what constitutes an effective immune response against the organism, this study aims to analyze functional immune differences in fish that are resistant/susceptible to provide information that could be used for the development of effective breeding programs.

2. Materials and Methods:

2.1 Fish

Forty full-sibling families of rainbow trout were produced at Lyndon Fish Hatcheries (Dundee, Ont.). These families were transported to the Hagen Aqua Lab or the Alma Aquaculture Facility at the University of Guelph as eyed eggs. Susceptibility to *F. psychrophilum* experimental infection was compared between the 40 family groups. Six to ten families per experiment were divided into four 60 L tanks containing 40 fish (40 g each) per tank, which were supplied with single pass well-water at 11^{o}C. Fish were fed a commercial pellet diet (Martin Mills Inc.). All maintenance and procedures were done in accordance with the University of Guelph Animal Care Guidelines.

2.2 Culture and quantification of F. psychrophilum strain 101

Frozen stocks of *F. psychrophilum* strain 101 (FPG 101) were sub-cultured onto cytophaga agar (CA) then following incubation at 15^{o}C for 72 h, cells were harvested and suspended in cytophaga broth (CB, Hesami et al., 2011). Cell suspensions were adjusted to an optical density of 0.6 at 600 nm using a Novaspec Plus spectrophotometer (GE Healthcare Life Sciences) and the suspension was then diluted to obtain a final

concentration of ~1 x 10^9 colony forming units (CFU)/mL. The concentration was then adjusted so that the desired CFU/mL for each trial could be obtained. To confirm the cell numbers, suspensions were enumerated by plating 0.1 mL of ten-fold serial dilutions on CA plates in triplicate. After incubation at 15^{o}C for 4 d, plates containing 25-250 colonies were counted and the number of CFU/mL calculated.

2.3 Determining susceptibility/resistance to F. psychrophilum (Preliminary Trials)

The Ontario *F. psychrophilum* isolate, FPG 101, was used for all experimental infections as it had been shown to produced ~50% mortality in experimental trials of 30-40 g rainbow trout at doses similar to those used in the current study (JS Lumsden pers. comm.). After sedation with ~50 ppm benzocaine (Millipore Sigma) in well-aerated water, triplicate tanks (n = 40) of each family received intraperitoneal (i.p.) 0.1 mL injections ranging in concentration between 1.13 and 6.60 x 10^7 CFU/mL. With every family, a fourth tank of fish was sham-injected with 0.1 mL of sterile CB as a negative control. Following injection, all fish were observed three times a day and any mortalities were collected and saved at -20°C. In addition, fish with marked exopthalmia, hemorrhage or skin ulcerations were removed and euthanized using an overdose of benzocaine. These fish were counted as mortalities.

After the predetermined trial period (15 d), any surviving fish were euthanized with an overdose of benzocaine and were recorded as survivors. The caudal fins of mortalities and final survivors were collected, submerged in RNAlater and stored at -20^{o}C for later use. Mortality was the measure used to rank the rainbow trout families for their resistance to *F. psychrophilum* infection. For subsequent experiments described below (sections **2.4** and **2.5**), resistant and susceptible families were selected based on this resistance ranking.

2.4 MH Class II β1 Genotyping

2.4.1 DNA Extraction

Based on the susceptibility results (section **2.3**), caudal fins from survivors and mortalities of families 3, 4, 6, 7, 9, and 10 were collected for DNA extraction. DNA was extracted from all fin clips as described by

Kellendonk, 2014. The final concentration of DNA was determined using the Take3 plate in a Synergy H1 plate reader (BioTek Instruments) before samples were moved to the -80^{o}C for storage.

2.4.2 Amplification and Sequencing of MH Class II ß1 genotypes

Primers were designed to amplify the MH class IIβ region for this study. The forward primer, OMYF (5'-TGCCAATTGCCTTCTACATTTGCCTG-3') was designed to a conserved region of MH class IIβ at the beginning of exon 1, while the reverse primer, OMYR (5'-TGGGGGCTCAACTGTCTTGTCCAGT-3') was designed to a conserved region at the end of exon 2 (Hodgins, 2011). These primers ensured the amplification of a portion of the peptide binding groove, the region that binds specifically to foreign antigens such as extracellular pathogens. For MH class IIβ amplification, the following PCR parameters were used: denaturation at 95^{o}C for 2 min, followed by 24 cycles of denaturation at 94^{o}C for 30 sec, annealing at 55^{o}C for 30 sec and extension at 72^{o}C for 1.5 min. A final extension at 72^{o}C was carried out for 8 min. Two PCR reactions were performed per individual. The amplified PCR products were separated on 1% agarose gels containing 3% Gel Red (Biotium Inc.). Bands of the desired size were cut out of the gel and extracted using the QIAquick Gel extraction kit (Qiagen). The resultant fragments were cloned into pGEM®-T Easy as per the manufacturer's instructions (Promega Corporation, Madison, WI). Competent *E. coli* XL1–blue cells were transformed using the Inoue procedure as described in Green and Sambrook, (2012) and grown on LB plates with 100 µg/mL of ampicillin, 240 ng/mL IPTG and 100 µg/mL X-gal. Ten colonies per PCR reaction were selected and grown in LB medium. Plasmids were extracted from the transformants using the GenElute Plasmid Miniprep Kit (Millipore Sigma) according to the manufacturer's instructions. Plasmids were sequenced using both T7 and SP6 primers at the TCAG sequencing facility (Sick Kids Hospital, Toronto, Ont.). The sequences were analyzed using both GENEious and the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A G-test was performed on the resulting contingency table using the SPSS statistical software. Significant differences were established at $p< 0.05$.

2.5 Induction of F. psychrophilum Antibody Responses

2.5.1 Infection

Two trials were performed to measure the levels of IgM antibody developed towards *F. psychrophilum* throughout infection. These trials were conducted in triplicate tanks (n = 40) when the fish were, on average, 108 g. The first trial (referred to as Trial 1) included two resistant families (F21 and F30), as well as two susceptible families (F11 and F17). After sedation with benzocaine, all fish received 100 µl injections i.p. of 1.10×10^7 CFU/mL of FPG101 suspended in CB. As a control, fish from the four families analyzed were mixed, then received 100 µl i.p. injections of sterile CB and separated into triplicate tanks. On days 0, 1, 7, 14, 21 and 28, blood was drawn from the caudal vein after anesthesia with ~50 ppm benzocaine. Fish were then euthanized using an overdose of benzocaine and whole-body weights and lengths were measured. Blood samples were left to clot overnight at 4°C before centrifugation at 1000 g for 10 min. The serum was then removed, aliquoted into 1.5 mL tubes and immediately stored at -20°C.

Because it has been reported that rainbow trout require up to 12 weeks to reach peak serum IgM levels when injected with killed *F. psychrophilum* (LaFrentz et al. 2002), a second trial (referred to as Trial 2) was conducted wherein fish were injected i.p. with 100 µl of a lower dose of live FPG101 (3.25×10^6 CFU/mL) and sampled over 140 days. In this trial, one resistant family (F32) and one susceptible family (F38) was used and all experiments were done in triplicate tanks (n = 40) where the fish were, on average, 85 g. For both families, triplicate control tanks were set up where all control fish received 100 µl i.p. injections of sterile CB broth. Serum samples were collected as described above. In Trial 2, the anterior head kidney was also removed from sampled individuals so that respiratory burst activity of head kidney leukocytes could be measured (described further in section **2.6**).

2.5.2 Isolating Antigens from F. psychrophilum

Subcultures of glycerol stocks of FPG101 were grown on CA at 14°C and carefully checked for purity. An isolated colony was then used to inoculate 50 mL of CB, which was subsequently used to inoculate 200 mL of CB after three days of incubation at 14°C. When the culture reached an optical density of approximately 0.8 at 525 nm, indicating a concentration of ~1 x 10^8 CFU/mL (Holt, 1987), 0.5% formalin was added to the flask and cells were incubated at 14°C for 24 h with gentle stirring. The killed bacterial cells were then washed twice with 4 mL of phosphate buffered saline (PBS) then harvested by centrifugation (9,500 g at 4°C for 15 min) and the resultant pellets resuspended in 2 mL of PBS.

Six hundred microliters of 0.1 mm zirconia/silica beads (BioSpec Products) was added to a 1.5 mL screw cap tube followed by the addition of 600 µl of the inactivated *F. psychrophilum* culture. The tubes were placed in a BioSpec mini-beadbeater and agitated on full speed for 45 sec followed by 1 min of incubation on ice. This procedure was repeated five times for each tube before the samples were centrifuged at 9,500 g for 15 min. Following centrifugation, the supernatants were removed and combined in a sterile 15 mL Falcon tube. The protein concentration of the bacterial lysate was then quantified using a bicinchoninic acid assay (ThermoFisher Scientific) following the manufacturer's protocol. The bacterial lysate was then diluted to a final concentration of 0.5 µg/mL in coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6).

2.5.3 Indirect ELISA Assay to Detect Levels of F. psychrophilum antibodies in Serum

One-hundred microliters of the whole-cell bacterial lysate preparation was used to coat the test wells of Nunc™ MicroWell™ 96-well microplates (ThermoFisher Scientific). Hyperimmune serum from rainbow trout injected with *F. psychrophilum* FPG25 (Hesami et al. 2008) was also used at a dilution of 1:50 as a positive control. To obtain hyperimmune serum, 200 g rainbow trout were injected in the dorsal sinus with 100 µl of formalin-killed FPG25. Three months later, fish received a 100 µl booster of formalin-killed *F. psychrophilum* FPG25. Serum samples were obtained at 5 months and 7 months following the initial exposure. Negative

control wells were coated with the whole cell bacterial lysate but were incubated with 100 µl Tris-buffered saline supplemented with 0.5% Tween 80 (TBS-T) rather than rainbow trout serum to account for any non-specific binding of the detecting antibodies. The coated plates were incubated overnight at 4^{o}C to enable adherence of the antigens.

The following day, all wells were washed three times with TBS-T. Three hundred microliters of blocking solution (2% laboratory grade gelatin (ThermoFisher Scientific) dissolved in TBS-T) was added to each well and incubated for 1 h at 37^{o}C. The plate was then washed three times with TBS-T and 100 µl of each trout serum (diluted 1:50) was added to triplicate test wells. After incubation for 1 h at room temperature (RT) the wells were washed thrice with TBS-T and 100 µl of mouse anti-rainbow trout IgM monoclonal antibody (Aquatic Diagnostics) at a dilution of 1:50 was added to each test well and hyperimmune serum control wells. The plate was incubated for 1 h at RT after which all wells were washed thrice with TBS-T. All test and the hyperimmune serum positive control wells received 100 µl of goat anti-mouse IgG conjugated to alkaline phosphatase (Millipore Sigma) at a dilution of 1:30 000. The plate was incubated for 1 h at RT and then washed three times with TBS-T. Fifty microliters of substrate, p-nitrophenyl phosphate (p-NPP), was added to each well and the plate was incubated in the dark for 30 min. The reaction was then stopped by the addition of 50 µl of 0.03M of NaOH to each well. The plate was read immediately at an absorbance of 405 nm in a Synergy H1 plate reader (BioTek). The negative control wells, which received TBS-T rather than trout serum, were averaged and subtracted from all other absorbance values. Plate variation was accounted for by normalizing each plate to the hyperimmune serum positive control. Statistical analysis was conducted using a two-way ANOVA through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA) where each of the triplicate tanks was considered an experimental unit (n = 3), and variation among tanks of each family receiving the same treatment was used as experimental error. This was then followed by Sidak's Multiple Comparisons Test with significant differences established at $p < 0.05$.

2.6 Measuring Respiratory Burst Activity (RBA) of Blood and Head Kidney Leukocytes

2.6.1 Infection

To determine whether RBA was associated with the observed resistance or susceptibility to *F. psychrophilum*, two infection trials were conducted. The families used for the first RBA trial are described above in section **2.5.1** (Trial 2). On days 0, 1, 7, 14 and 28, three fish per tank were euthanized with an overdose of benzocaine and the anterior head kidneys were collected and transferred to sterile plasticware containing approximately 20 mL of L-15 medium (Lonza) supplemented with 5% FBS, 100 U/mL of penicillin and 100 U/ml of streptomycin. These samples were stored on ice until the HKL could be isolated.

To validate the results observed for RBA in Trial 2, a repeat trial (Trial 3) was completed using the resistant family, F36 and the susceptible family, F38. Challenged fish received 100 µl i.p. injections of FPG101 (3.50×10^6 CFU/mL). This trial was conducted in triplicate tanks (n = 40) when the fish were, on average, 200 g. As a control, fish from the two families analyzed were mixed, received 100 µl i.p. injections of sterile CB, and were separated into triplicate tanks. On days 0, 3, 7, 14 and 20, three fish per tank were euthanized with an overdose of benzocaine so that the anterior head kidney of each individual could be removed and processed as described.

2.6.2 Isolation of Head Kidney Leukocytes

The head kidney samples were vigorously vortexed 5 times at maximum speed for 30-60 sec to dissociate the cells from the underlying connective tissues. The dissociated cells were passed through a 40 µm sterile nylon mesh (ThermoFisher Scientific) then rinsed with 5 mL of sterile media. The resulting cell suspensions were brought up to a final volume of 28 mL and, after thorough mixing, divided into four 7 mL aliquots. The 7 mL cell suspensions were then layered on top of 3 mL of Histopaque 1077 (Millipore Sigma) and centrifuged for 500 x g for 30 min at 4°C. The leukocyte layer located at the medium:histopaque interface was collected and cells were pelleted by centrifugation at 500 x g for 5 min at 4°C. The supernatant was

aspirated and the pellets were washed using 5 mL of PBS and again, harvested by centrifugation. The resultant cell pellets were resuspended in 1-3 mL of fresh 5% FBS/L-15 so that cell concentration and viability could be determined using a trypan blue exclusion assay. After cell viability was determined to be above 95%, cell concentrations were adjusted to 7.0 x 10^6 cells/mL.

2.6.3 Real-time luminol-enhanced chemiluminescence assay for RBA

The RBA of both the whole blood samples and the head kidney leukocytes (HKL) was measured following the protocol of Jimenez-Vera et al. (2013) with minor variations. Briefly, Corning™ 96-well solid white polystyrene microplates were prepared with 6 wells containing either 100 µl of HKL (7.0 x 10^6 cells/mL) or 75 µl of blood sample from each individual. The first three wells per individual received 40 µl of luminol (10 mM, Millipore Sigma), 100 µl of zymosan A (20 mg/mL, Millipore Sigma), and were brought to a final volume of 300 µl using Hank's Balanced Salt Solution (HBSS, Millipore Sigma). To ensure that the increase in luminescence was due to stimulation, the remaining three wells per individual were used as unstimulated controls and received 100 µl of HBSS in place of zymosan A. The chemiluminescence of each well was measured with a Synergy H1 luminometer (Biotek) every 2 min for 90 min. The chemiluminescence emission of the HKL and blood samples was expressed as the integral of the relative light units (RLU) between 0 and 90 min. Statistical analysis was conducted using a two-way ANOVA through the GraphPad Prism software (v7.0, GraphPad Software, Inc. USA) where each of the triplicate tanks was considered an experimental unit (n = 3), and variation among tanks of each family receiving the same treatment was used as experimental error. This was followed by Sidak's Multiple Comparisons Test and significant differences were established at $p < 0.05$.

3. Results and Discussion

3.1 Preliminary trials to determine rainbow trout families resistant/susceptible to F. psychrophilum

Fish from 40 families were infected with *F. psychrophilum* and survival was monitored. As **Figure 1A** displays, there was a broad range of survival between the families, with the most resistant family (F8) having 90.9% survival while the most susceptible family (F36) had only 2.5% survival. This broad range in BCWD susceptibility has been observed in previous studies aimed at understanding and consistently standardizing the production of resistant rainbow trout (Hadidi et al. 2008, Wiens et al. 2013). From the results presented in this study, families were chosen to conduct repeat infection trials so that both genetic (families presented in **Figure 1B**) and functional immune markers (families presented **Figure 1C**) could be assessed. To ensure that the observed resistance/susceptibility was consistent, the infection trial was repeated for a resistant family (F7) and a susceptible family (F10). In the initial trial F7 and F10 displayed 90.5% and 14.3% survival respectively. These results were confirmed to be robust as F7 and F10 displayed 86.0% and 19.9% survival respectively when the infection trial was repeated (data not shown). As the fish were approximately the same size (~30-50 g) during this repeat trial, these results provide evidence that susceptibility to FPG101 was consistent at this size/age.

3.2 Relating MH class IIβ genotype to disease susceptibility

To explore whether there is a link between BCWD resistance and MH class IIβ alleles, a minimum of 18 individuals from six of the rainbow trout families studied were genotyped. The families selected (F3, F4, F6, F7, F9 and F10) had variable survival rates when challenged with *F. psychrophilum* (see **Figure 1B**) and thus were hypothesized to have variability in their MH genotypes. **Table 1** presents the MH class IIβ genotype frequencies observed between the six different families. Overall, there were five alleles identified which created a total of twelve MH genotypes in the six families studied. This is a lower number than previously observed when wild fish populations were analyzed (Conejeros et al. 2012, Conejeros et al. 2008, Dorschner

et al. 2000). However, because the rainbow trout families used in the current study were not from wild populations and thus had likely been selected for faster growth, better feed to weight conversion and better flesh quality, this greater degree of homogeneity was not unexpected. Increasing the variability of MH genotypes has not been a breeding focus for these families, therefore low diversity in MH genes may simply be a by-product of selection for other desirable traits. This has been reported in many other studies of production (Gomez et al. 2011, Croisetiere et al. 2008, Kjoglum et al. 2007, Grimholt et al. 2003), thus the low allele frequencies presented in this study are likely representative of the commercial origin of the families that were used.

Remarkably, individuals with three or four allele genotypes for MH class IIβ were observed in this study (**Table 1**) but these generally occurred at very low frequencies in the families analyzed. The occurrence of two or more alleles per individual has been observed in previous MH studies (Conejeros et al. 2012, Conejeros et al. 2008, Kruiswijk et al. 2004, Noakes et al. 2003, Dixon et al. 1996) but this was usually attributed to high MH polymorphism. Although PCR artifacts and false alleles can arise due to the nature of MH sequences and the cloning process, precautions were taken in this study by modifying the PCR protocol to minimize their formation (Lenz and Becker, 2008). Accordingly, we have high confidence that the sequences presented in this study represent real genotypes, even those animals containing three to four alleles. Another explanation for these interesting genotypes is that the alleles come from two different loci. If this is the case, after phylogenetic tree analysis, researchers would anticipate seeing two major branches with clusters of alleles in each. Instead, researchers that have analyzed this phenomenon have observed a subdivided tree with many small clusters of alleles (Dorschner et al. 2000). As this has not been the central focus of MH analysis in teleost species, further research is required.

When relating the allele prevalence to the observed number of survivors per family, as presented in **Table 2**, both DAB*1001/DAB*1001 and DAB*1001/DAB*0801 were observed at the highest frequency in both survivors and mortalities. These results are consistent with what has been previously observed in

microsatellite and SNP analysis studies wherein classical MH class II genotypes were not found to be linked to BCWD resistance (Johnson et al. 2008) or only a weak correlation ($p < 0.15$) has been noted (Overturf et al. 2010). Despite these similar results, the MH genotypes presented in the current study represent full allele sequences rather than correlating a measure of MH genetic diversity with BCWD resistance. As a result, this is the first study in which specific amino acid sequences of the MH class IIβ peptide binding groove have been analyzed in rainbow trout to determine whether they are associated with resistance/susceptibility to *F. psychrophilum* infection. Based on the analysis of 110 rainbow trout from six different full-sibling families, the MH class II genotypes of these fish do not appear to be associated with resistance/susceptibility towards FPG101.

3.3 IgM antibody development in rainbow trout families resistant/susceptible to F. psychrophilum

Although the pathogenesis of *F. psychrophilum* is not fully understood, the organism is known to have a systemic phase during infection (Nilsen et al. 2011, Starliper, 2011). Accordingly, it is reasonable to hypothesise that the humoral immune response, including antibody production, may be important for the control of BCWD. In a recent study by Marancik et al. (2015) comparing whole body transcriptomes between resistant and susceptible rainbow trout groups, higher *igm* gene transcript levels were observed in rainbow trout lines that were resistant when compared to their susceptible counterparts. Thus, serum IgM could play a role in the observed resistance to BCWD. In the current study, the production of rainbow trout serum IgM against *F. psychrophilum* FPG101 appeared to increase over time regardless of the resistance in the six families studied. In Trial 1, anti-*F. psychrophilum* IgM levels increased over the 28-day infection but there was no significant difference in antibody production between the resistant (F21 and F30) and the susceptible (F11 and F17) families (**Figure 2A**). A similar trend was also seen in the 140-day infection trial, Trial 2 (**Figure 2B**), where serum IgM levels were also observed to increase over time with no significant difference between the resistant (F39) and susceptible (F32) families studied. In contrast, the saline-injected controls in Trial 2

showed no increase in anti-*F. psychrophilum* serum IgM levels over time. Infection with *F. psychrophilum* induces an antibody response, but the levels were not significantly different in resistant versus susceptible fish. Although there has been an increasing interest in understanding the mucosal and systemic immunity in response to *F. psychrophilum* (Makesh et al., 2015), this is the first study in which serum IgM levels have been compared families with established *F. psychrophilum* resistance and susceptibility phenotypes.

The survival curves of families in both Trial 1 (**Figure 2C**) and Trial 2 (**Figure 2D**) did not reveal significant differences between resistant and susceptible families in contrast to the preliminary trials (**Figure 1C**). This can be seen in **Figure 2E** and **2F** where the percent mortality of families in the preliminary trial is compared to the percent mortality in Trial 1 (**Figure 2E**) and Trial 2 (**Figure 2F**). In the preliminary trials, the resistant families had low mortality rates (5 to 42%) while the rates in susceptible families were much higher (57 to 85%). However, upon completion of Trial 1 and Trial 2, the percent mortality was more similar regardless of whether the family was previously observed to be resistant or susceptible to *F. psychrophilum* (**Figure 2E** and **2F**). In Trial 1, the four families studied had observed mortalities between 70 and 80% while in Trial 2, the two families studied had mortalities of 25 and 30%. The difference in mortality between Trial 1 and Trial 2 can be explained by the fact that the infectious dose used in Trial 2 was lower by a factor of ~10 which was used to ensure enough fish survived for sampling throughout the longer (4 mo.) period of this trial. The large differences in mortality between Trials 1 and 2 also reflected differences in the size and age of the fish. In the preliminary trials, all fish were approximately 40 g in size, versus the 108 g or 85 g average size of the fish for Trials 1 and 2 respectively. These results further support previous findings that the age of the host is very important in *F. psychrophilum* infection. For example, this age-related resistance has been observed in RTFS wherein macrophages obtained from susceptible 10-week-old rainbow trout fry were shown to contain viable, potentially replicating *F. psychrophilum,* while resilient fish aged 5 months and older did not (Decostere et al. 2001). Age-related disease susceptibility in salmonids has been observed for several other pathogens including the parasite *Hexamita salmonis* (Tojo and Satamarina, 1998), infectious pancreatic

necrosis virus (IPNV, Espinoza and Kuznar, 2002, Roberts and Pearson, 2005) and *Flavobacterium branchiophilum* (Good et al. 2008). Thus, age/size differences have been shown to influence susceptibility to several aquatic diseases including *F. psychrophilum*, which may provide an explanation as to why the observed serum IgM for Trials 1 and 2 did not correlate with the resistance/susceptibility observed in the preliminary trial.

3.4 The Effect of Head Kidney and Total Blood RBA on resistance to F. psychrophilum infection

The RBA of head kidney leukocytes from the infected resistant (F39) and susceptible (F32) families of Trial 2 were observed to differ significantly on day 14 (**Figure 3A**). Otherwise there was no significant difference in RBA between the groups. Throughout infection, the two families had very similar trends in RBA with high activity observed on Days 0 and 1, followed by a significant decrease in activity on Day 7 of the trial. For the final two weeks of the trial, RBA was observed to slowly recover until it was restored to pre-infection levels on Day 28 (**Figure 3A**). The large drop in RBA on Day 7 was interesting as this was also the time when mortalities were first observed in Trial 2. On Day 28 and beyond, when RBA activity appeared to have fully recovered, mortalities were rarely observed. The observed decrease in RBA of HKL in infected fish appears to be a consequence of *F. psychrophilum* infection as the sterile CB infected control fish had no significant difference in HKL RBA across all time points (**Figure 3A**).

Depending on the organism in question, bacterial pathogens influence the respiratory burst activity of fish leukocytes in a variety of ways. The intracellular pathogen, *Aeromonas hydrophila* has been shown to increase the RBA of host leukocytes 7 days following infection (Biller-Takahashi et al. 2013). In contrast, both intracellular *Renibacterium salmoninarum* and extracellular *Vibrio anguillarum* have been shown to reduce the spleen leukocyte and HKL RBA of their hosts (Densmore et al. 1998, Sepulcre et al. 2007). This is not surprising considering that *R. salmoninarum* and *V. anguillarum* both produce virulence factors that negatively impact fish leukocytes (p57 and RTX proteins respectively, Wiens and Kaattari, 1991, Li et al.

2008), yet a comparable virulence factor of *F. psychrophilum* has not yet been discovered. To date there is little to no data regarding the effect of *F. psychrophilum* on total leukocyte populations but it has been previously shown that the organism can survive within, and be cytotoxic to, rainbow trout phagocytes (Nematollahi et al. 2005, Decostere et al. 2001, Wiklund and Dalsgaard, 2003, Lammens et al. 2000). As HKL would contain phagocytes and the RBA of these cells was dramatically reduced in this study (**Figure 3A** and **3B**), perhaps this cytotoxic effect to phagocytes extends to other leukocytes. Further research is necessary to understand what aspect of *F. psychrophilum* is responsible for the decrease in HKL RBA that was observed in this study.

To ensure that the observed reduction in RBA was due to bacterial challenge, rather than leukocytes leaving the head kidney to monitor the periphery, a repeat trial (Trial 3) was conducted in which the respiratory burst activity of the HKL as well as in total blood from each fish was measured. If leukocytes had left the head kidney to monitor the periphery, an increase in the RBA of total blood would be anticipated. Much like what was observed in Trial 2, the RBA of the HKL did not differ significantly between the resistant (F36) and susceptible (F38) families studied. A significant decrease in RBA was observed at Day 3 of Trial 3 (**Figure 3B**) and was maintained until trial completion on Day 20. Further, there was no difference in total blood RBA between the two families studied throughout infection (**Figure 3C**). It is tempting to speculate that the HKL are not travelling to the bloodstream because the total blood RBA did not significantly change throughout infection (**Figure 3C**). This is a possibility, but it is also feasible that immune cells were travelling to inflammatory sites such as the spleen or external lesions, and that their transit was not monitored at the time points selected for this trial. Although there is limited research on immune cell transit in salmonids, it has been shown in the embryonic zebrafish model that it can take as little as 4 hours for neutrophils to travel to the injection site of live *Pseudomonas aeruginosa* while no neutrophil transport is observed following sterile PBS injection (Deng et al. 2012). Similarly, phagocytes also migrate rapidly to sites of inflammation and bacterial infection (Herbomel et al. 1999, Davis et al. 2002, Zakrzewska et al. 2010) thus the time points

selected for this study may not have been early enough to observe leukocyte migration. The results of the current study do suggest that *F. psychrophilum* may reduce the respiratory burst activity of head kidney leukocytes but this finding merits further research to validate whether the leukocytes are truly influenced by *F. psychrophilum*, or simply in transit to deal with the infection at other locations.

Although there was little to no difference in the RBA between the resistant and susceptible families studied, it is important to relate these results to the survival of each group throughout *F. psychrophilum* infection. In the preliminary trial, low mortality was observed in the resistant families, F32 and F36 (2-5%), while high mortality was noted in the susceptible families, F38 and F39 (42-55%) (**Figure 1C**). Despite these initial large differences in survival, upon completion of the RBA trials, all four of the families had consistent mortalities ranging between 1-10% (**Figure 3D**). Considering that all four of these families had similar survival during the RBA trials, it is not surprising that the RBA would have little difference between the resistant and susceptible families as well. As discussed in section **3.3**, differences in fish size and age could explain why the immune performance does not better correlate with the resistance/susceptibility that was observed in the preliminary trials. Furthermore, the infectious dose was much higher in the preliminary trial compared to the final two immune function trials (Trials 2 and 3). This, along with the increased size/age of the fish, influenced the observed mortality but was essential for experimental design to ensure that some fish from all families survived until the end of each infection trial.

Conclusions

In this study, we showed that the resistance of rainbow trout fry to *F. psychrophilum* may be influenced by age/size as differences in susceptibility were not consistent at later life stages. Breeding for resistance in fry/fingerlings could still be beneficial for fish farmers as BCWD tends to be a disease of young salmonids. As such, losses of even young stock can be costly in terms of increased management costs, reduced feed conversion, etc. No clear genetic markers were identified that could be used for informed

breeding strategies aimed at combatting BCWD. More specifically, MH class IIβ genotypes do not appear to correlate with the observed resistance/susceptibility to *F. psychrophilum*. In addition, neither anti-*F. psychrophilum* IgM levels nor the RBA of HKL and total blood were found to differ between families previously observed to be resistant or susceptible to the organism. It is possible that the early observed differences in susceptibility would correlate with differences in these selected immune markers if tested during that earlier life stage. Accordingly, future research should emphasize the study of functional immune markers during the fry stage of development and additional immune/host markers should be evaluated. As RBA of HKL was significantly reduced in response to live infection with *F. psychrophilum*, future studies should also focus on understanding the pathogenesis of *Flavobacterium psychrophilum* FPG101 and its interaction with rainbow trout leukocytes.

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References:

1. Bebak, J.A., Welch, T.J., Starliper, C.E., Baya, A.M., Garner M.M., 2007. Improved husbandry to control an outbreak of rainbow trout fry syndrom caused by infection with *Flavobacterium psychrophilum*. *Journal of the American Veterinary Association,* 231, 114-116.
2. Biller-Takahashi, J.D., Takahashi, L.S., Saita, M.V., Gimbo, R.Y. and Urbinati, E.C., 2013. Leukocytes respiratory burst activity as indicator of innate immunity of pacu *Piaractus mesopotamicus. Barzilian Journal of Biology*, 73, 425-429.
3. Brown, L.L., Cox, W.T., Levine R.P., 1997. Evidence that the causal agent of bacterial cold-water disease *Flavobacterium psychrophilum* is transmitted within salmonid eggs. *Diseases of Aquatic Organisms*, 29, 213-218.
4. Callazos, M.E., Barriga, C., Ortega, E., 1994a. Enhanced granulocyte phagocytosis at low winter temperature and high summer temperature in tench (*Tinca tinca* L.). *Comp Biochem Physiol* 109, 643-648.
5. Callazos, M.E., Ortega, E., Barriga, C., 1994b. Effect of Temperature on the immune system of a cyprinid fish (*Tinca tinca*, L.). Blood phagocyte function at low temperature. *Fish and Shellfish Immunology* 4, 231-238.
6. Cipriano, R.C., Holt, R.A., 2005. *Flavobacterium psychrophilum*, cause of bacterial cold-water disease and rainbow trout fry syndrome. Fish Disease Leaflet No. 86. United States Dept. of the Interior. U.S. Geological Service, National Fish Health Research Laboratory, Leetown, WV
7. Conejeros, P., Phan, A., Power, M., Alekseyev, S., O'Connell, M., Dempson, B., Dixon, B., 2008. MH class II alpha polymorphism in local and global adaptation of Arctic charr (*Salvelinus alpinus L*.). *Immunogenetics*, 60, 325–37.
8. Conejeros, P., Power, M., Alekseyev, S., Dixon, B., 2012. Global major histocompatibility class II β (MH-IIβ)-polymorphism in Arctic charr *Salvelinus alpinus*. *Journal of Fish Biology*, 81(4):1158–74.

9. Croisetiere, S., Tarte, P.D., Bernatchez, L., Belhumeur, P., 2008. Identification of MHC class IIβ resistance/susceptibility alleles to *Aeromonas salmonicida* in brook charr (*Salvelinus fontinalis*). *Molecular Immunology*, 45, 3107-3116.

10. Crump, E.M., Burian, J., Allen, P.D., Kay, W.W., 2005. Identification and expression of a host-recognized antigen, FspA, from *Flavobacterium psychrophilum*. *Microbiology*, 151, 3127-3135.

11. Davis, J.M., Clay, H., Lewis, J.L., Ghori, N., Herbomel, P., Ramakrishnan, L., 2002. Real-time visualization of mycobacterium-macrophage interactions leading to initiation of granuloma formation in zebrafish embryos. *Immunity,* 17, 693-702.

12. Decostere, A., D'Haese, E., Lammens, M., Nelis, H., Haesebrouk, F., 2001. *In vivo* study of phagocytosis, intracellular survival and multiplication of *Flavobacterium psychrophilum* in rainbow trout, *Oncorhynchus mykiss* (Walbaum), spleen phagocytes. *Journal of Fish Diseases,* 24, 482-487.

13. Densmore, C.L., Smith, S.A., Holladay, S.D., 1998. *In vitro* effects of the extracellular protein of *Renibacterium salmoninarum*on phagocyte function in brook trout (*Salvelinus fontinalis*). *Veterinary Immunology and Immunopathology,* 62, 349-357.

14. Deng, Q., Harvie, E.A., Huttenlocher, A., 2012. Distinct signalling mechanisms mediate neutrophil attraction to bacterial infection and tissue injury. *Cellular Microbiology,* 14, 517-528.

15. Dionne, M., Miller, K.M., Dodson, J.J., Bernatchez, L., 2009. MHC standing genetic variation and pathogen resistance in wild Atlantic salmon. *Philosophical Transactions: Biological Sciences,* 364, 1555-1565.

16. Dixon, B., Nagelkerke, L., Sibbing, F., Egberts, E., Stet, R.J., 1996. Evolution of MHC class II beta chain-encoding genes in the Lake Tana barbel species of flock (*Barbus intermedius* complex). *Immunogenetics,* 44, 419-431.

17. Dorschner, M.O., Duris, T., Bronte, C.R., Burnham-Curtis, M.K., Phillips, R.B., 2000. High levels of MHC class II allelic diversity in lake trout from Lake Superior. *The Journal of Heredity*, 91, 359–63.

18. Dumetz, F., Duchaud, E., LaPatra, S.E., Le Marrec, C., Claverol, S., Urdaci, M., Le Henaff, M., 2006. A protective immune response is generated in rainbow trout by an OmpH-like surface antigen (P18) of *Flavobacterium psychrophilum*. *Applied Environmental Microbiology,* 72, 4845-4852.

19. Espinoza, J.C., Kuznar, J., 2002. Rapid simultaneous detection and quantitation of infectious pancreatic necrosis virus (IPNV). *Journal of Virological Methods,* 105, 81-85.

20. Fillatreau, S., Six, A., Magadan, S., Castro, R., Sunyer, J.O., Boudinot, P., 2013. The astonishing diversity of Ig classes and B cell repertoired in teleost fish. *Frontiers in Immunology* 4, 1-14.

21. Forthal, D.N., 2014. Functions of antibodies. *Microbiology Spectrum,* 2, 1-17.

22. Gjedrem, T., Salte, R., Gjoen, H.M., 1991. Genetic variation in susceptibility of Atlantic salmon to furunculosis. *Aquaculture* 97, 1-6.

23. Gomez, D., Conejeros, P., Consuegra, S., Marshall, S.H., 2011. MHC mediated resistance to *Piscirickettsia salmonis* in salmonids farmed in Chile. *Aquaculture*, 318, 15-19.

24. Gomez, E., Mendez, J., Cascales, D., Guijarro, J.A., 2014. *Flavobacterium psychrophilum* vaccine development: a difficult task. *Microbial Biotechnology,* 7, 414-423.

25. Good, C.M., Thorburn, M.A., Stevenson, M.W., 2008. Factors associated with the incidence of bacterial gill disease in salmonid lots reared in Ontario, Canada government hatcheries. *Preventive Veterinary Medicine,* 83, 297-307.

26. Green, M.R., Sambrook, J., 2012. "The Inoue method for preparation and transformation of competent *E. coli*: Ultracompetent cells" in Molecular Cloning: A laboratory manual 4th Ed. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, USA.

27. Grimholt, U., Larsen, S., Nordmo, R., Midtlyng, P., Kjoeglum, S., Storset, A., Saebo, S., Stet, R.J.M., 2003. MHC polymorphism and disease resistance in Atlantic salmon (*Salmo salar*); facing pathogens with single expressed major histocompatibility class I and class II loci. Immunogenetics, 55, 210–9.

28. Hadidi, S., Glenney, G.W., Welch, T.J., Silverstein, J.T., Wiens, G.D., 2008. Spleen size predicts resistance of rainbow trout to *Flavobacterium psychrophilum* challenge. *Journal of Immunology,* 180, 4156-4165.

29. Henryon, M., Berg, P., Oleson, N.J., Kjae, T.E., Slierendrecht, W.J., Jokumsen, A., Lund, I. 2005. Selective breeding provides an approach to increase resistance of rainbow trout (*Oncorhynchus mykiss*) to the diseases, enteric redmouth disease, rainbow trout fry syndrome and viral haemorrhagic septicaemia. *Aquaculture* 250, 621-636.

30. Herbomel, P., Thisse, B., Thisse, C., 1999. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development,* 126, 3735-3745.

31. Hesami, S., Allen, K.J., Metcalf, D., Ostland, V.E., MacInnes, J.I., Lumsden, J.S., 2008. Phenotypic and genotypic analysis of *Flavobacterium psychrophilum* from Ontario salmonids with bacterial coldwater disease. *Canadian Journal of Microbiology*, 54, 619-629.

32. Hesami, S., Metcalf, D.S., Lumsden, J.S., MacInnes, J.I., 2011. Identification of cold-temperature-regulated genes in *Flavobacterium psychrophilum*. *Applied and Environmental Microbiology,* 77, 1593-1600.

33. Holt, R.A., 1987. *Cytophaga psychrophila the causative agent of bacterial cold-water disease in salmonid fish.* PhD Thesis, Corvalis, OR: Oregon State University.

34. Hodgins, S., 2011. *Characterizing the immune response of Flavobacterium psychrophilum, and possible resistance/susceptibility conferring major histocompatibility alleles in Oncorhynchus mykiss.* MSc Thesis, Waterloo, ON: University of Waterloo.

35. Jimenez-Vera, N.I., Pietretti, D., Wiegertjes, G.F., Nielsen, M.E., 2013. Comparative study of β-glucan induced respiratory burst measured by nitroblue tetrazolium assay and real-time luminol-enhanced chemiluminescence assay in common carp (*Cyprinus carpio* L.). *Fish and Shellfish Immunology,* 34, 1216-1222.

36. Johnson, N.A., Vallejo, R.L., Silverstein, J.T., Welch, T.J., Wiens, G.D., Hallerman, E.M., Palti, Y., 2008. Suggestive association of major histocompatability IB genetic markers with resistance to bacterial cold water disease in rainbow trout (*Oncorhynchus mykiss*). *Marine Biotechnology,* 10, 429-437.

37. Kellendonk, C., 2014. *Identification of MH class IIβ alleles conferring resistance/susceptibility to Flavobacterium psychrophilum in Oncorhynchus mykiss families.* MSc Thesis, Waterloo, ON: University of Waterloo.

38. Kjoglum, S., Larsen, S., Bakke, H.G., Grimholt, U., 2006. How specific MHC class I and class II combinations affect disease resistance against infectious salmon anaemia in Atlantic salmon (*Salmo salar*). *Fish and Shellfish Immunology* 21, 431-441.

39. Kjoglum, S., Larsen, S., Bakke, H.G., Grimholt, U., 2007. The effect of specific MHC class I and class II combinations on resistance to furunculosis in Atlantic salmon (*Salmo salar*). *Scandanavian Journal of Immunology*, 67, 160-168

40. Kjoglum, S., Henryon, M., Aasmundstad, T., Korsgaard, I., 2008. Selective breeding can increase resistance of Atlantic salmon to furunculosis, infectious salmon anaemia and infectious pancreatic necrosis. *Aquaculture Research,* 39, 498-505

41. Kruiswijk, C.P., Hermsen, T., Fujiki, K., Dixon, B., Savelkoul, H.F.J., Stet, R.J.M., 2004. Analysis of genomic and expressed major histocompatibility class Ia and class II genes in a hexaploid Lake Tana African “large” barb individual (*Barbus intermedius*). *Immunogenetics,* 55, 770-781.

42. LaFrentz, B.R., LaPatra, S.E., Jones, G.R., Congleton, J.L., Sun, B., Cain, K.D., 2002. Characterization of serum and mucosal antibody responses and relative percent survival in rainbow trout, *Oncorhynchus mykiss* (Walbaum), following immunization and challenge with *Flavobacterium psychrophilum. Journal of Fish Diseases,* 25, 703-713.

43. Lammens, M., Decostere, A., Haesebrouk, F., 2000. Effect of *Flavobacterium psychrophilum* strains and their metabolites on the oxidative activity of rainbow trout *Oncorhynchus mykiss* phagocytes. *Diseases of Aquatic Organisms,* 41, 173-179,

44. Lenz, T.L., Becker, S., 2008. Simple approach to reduce PCR artefact formation leads to reliable genotyping of MHC and other highly polymorphic loci—implications for evolutionary analysis. *Gene*, 427, 117-123

45. Li, L., Rock, J.L., Nelson, D.R., 2008. Identification and characterization of a Repeat-in-Toxin gene cluster in *Vibrio anguillarum*. *Infection and Immunity,* 76, 2620-2632.

46. Lumsden, J.S., Young, K., Welsh, K., MacInnes, J., Russell, S., Hesami, S., 2004. Management Approaches for Coldwater Diseases caused by Flavobacterium psychrophilum. *Proceedings of the Canadian Freshwater Aquaculture Symposium.* Aquaculture Canada, 11, 111-116.

47. Marancik, D., Gao, G., Paneru, B., Ma, H., Hernandez, A.G., Salem, M., Yao, J., Palti, Y., Wiens, G.D., 2015. Whole-body transcriptome of selectivecly bred, resistant-, control- and susceptible-line rainbow trout following experimental challenge with *Flavobacterium psychrophilum*. *Frontiers in Genetics,* 453, doi:10.3389/fgene.2014.00453.

48. Makesh, M., Sudheesh, P.S., Cain, K.D., 2015. Systemic and mucosal response of rainbow trout to immunization with an attenuated *Flavobacterium psychrophilum* vaccine strain by different routes. *Fish and Shellfish Immunology,* 44, 156-163.

49. Meyer, F.P., 1991. Aquaculture disease and health management. *Journal of Animal Science* 69, 4201-4208.

50. Neefjes, J., Jongsma, M.L.M., Paul, P., Bakke, O., 2011. Towards a systems understanding of MHC class I and MHC class II antigen presentation. *Nature Review Immunology,* 11, 823-836.

51. Nematollahi, A., Decostere, A., Pasmans, F., Haesebrouck, F., 2003. *Flavobacterium psychrophilum* infections in salmonid fish. *Journal of Fish Diseases*, 26, 563-574.

52. Nematollahi, A., Pasmans, F., Haesebrouck, F., Decostere, A., 2005. Early interactions of *Flavobacterium psychrophilum* with macrophages of rainbow trout *Oncorhynchus mykiss*. *Diseases of Aquatic Organisms,* 64, 23-28.

53. Nikoskelainen, S., Bylund, G., Lilius, E., 2004. Effect of environmental temperature on rainbow trout (*Oncorhynchus mykiss*) innate immunity. *Developmental and Comparative Immunology* 28, 581-592.

54. Nilsen, H., Olsen, A.B., Vaagnes, O., Hellberg, H., Bottolfsen, K., Skjelstad, H., Colquhoun, D.J., 2011. Systemic *Flavobacterium psychrophilum* infection in rainbow trout, *Oncorhynchus mykiss* (Walbaum), farmed in fresh and brackish water in Norway. *Journal of Fish Diseases,* 34, 403-408.

55. Noakes, M., Reimer, T., Phillips, R.B., 2003. Genotypic characterization of an MHC class II locus in lake trout *(Salvelinus namaycush)* from Lake Superior by single-stranded conformational polymorphism analysis and reference strand-mediated conformational analysis. *Marine Biotechnology (New York, N.Y.)*, 5, 270-278.

56. Overturf, K., LaPatra, S., Towner, R., Campbell, N., Narum, S., 2010. Relationships between growth and disease resistance in rainbow trout, *Oncorhynchus mykiss* (Walbaum). *Journal of Fish Diseases,* 33, 321-329.

57. Rakus, K.L., Wiegertjes, G.F., Jurecka, P., Walker, P.D., Pilarczyk, A., Irnazarow, I., 2009. Major histocompatibility (MH) class II B gene polymorphism influences disease resistance of common carp (*Cyprinus carpio* L.). *Aquaculture,* 288, 44-50.

58. Reyes-Cerpa, S., Maisey, K., Reyes-Lopez, F., Toro-Ascuy, D., Sandino, A.M., Imarai, M., 2013. Fish cytokines and immune response. In *New Advances and Contributions to Fish Immunology*, by H. Turker, pp 3-58. InTech.

59. Roberts, R.J., Pearson, M.D., 2005. Infectious pancreatic necrosis in Atlantic salmon *Salmo salar* L. *Journal of Fish Diseases,* 28, 383-390.

60. Sepulcre, M.P., Sarropoulou, E., Kotoulas, G., Meseguer, J. and Mulero, V., 2007. *Vibrio anguillarum* evades the immune response of the bony fish sea bass (*Dicentrarchus labrax* L.) through the inhibition of leukocyte respiratory burst and down-regulation of apoptotic caspases. *Molecular Immunology,* 44, 3751-3757.

61. Silverstein, J.T., Vallejo, R.L., Palti, Y., Leeds, T.D., Rexroad, C.E. 3rd, Welch, T.J., Wiens, G.D., Ducrocq, V., 2009. Rainbow trout resistance to bacterial cold-water disease is moderately heritable and is not adversely correlated with growth. *Journal of Animal Science,* 87, 860-867.

62. Starliper, C.E., 2011. Bacterial coldwater disease of fishes caused by *Flavobacterium psychrophilum*. *Journal of Advanced Research,* 2, 97-108.

63. Sudheesh, P.S., LaFrentz, B.R., Call, D.R., Siems, W.F., LaPatra, S.E., Wiens, G.D., Cain, K.D., 2007. Identification of potential vaccine target antigens by immunoproteomic analysis of a virulent and non-virulent strain of the fish pathogen *Flavobacterium psychrophilum. Diseases of Aquatic Organisms,* 74, 37-47.

64. Sunyer, J.O., 2013. Fishing for mammalian paradigms in the teleost immune system. *Nature Immunology,* 14, 320-326.

65. Tojo, J.L., Santamarina, M.T., 1998. Oral pharmacological treatments for parasitic diseases of rainbow trout *Oncorhynchus mykiss*. I: *Hexamita salmonis*. *Diseases of Aquatic Organisms,* 33, 51-56.

66. Wiens, G.D., Kaattari, S.L., 1991. Monoclonal antibody characterization of a leucoagglutinin produced by *Renibacterium salmoninarum*. *Infection and Immunity,* 59, 631-637.

67. Wiens, G.D., LaPatra, S.E., Welch, T.J., Evenhuis, J.P., Rexroad, C.E., Leeds, T.D., 2013. On-farm performance of rainbow trout (*Oncorhynchus mykiss*) selectively bred for resistance to bacterial coldwater disease: Effect of rearing environment on survival phenotype. *Aquaculture,* 388-391, 128-136.

68. Wiklund, T., Dalsgaard, I., 2003. Association of *Flavobacterium psychrophilum* with rainbow trout (*Oncorhynchus mykiss*) kidney phagocytes *in vitro*. *Fish and Shellfish Immunology,* 15, 387-395.

69. Wood, E.M., Yasutake, W.T., 1956. Histopathology of fish III. Peduncle ('coldwater') disease. *Progressive Fish-Culturist,* 18, 58-61.

70. Uribe, C., Folch, H., Enriquez, R., Moran, G., 2011. Innate and adaptive immunity in teleost fish: A review. *Veterinarni Medicina,* 56, 486-503

71. Xu, T., Chen, S., Zhang, Y., 2010. MHC class IIα gene polymorphism and its association with resistance/susceptibility to *Vibrio anguillarum* in Japanese flounder (*Paralichthys olivaceus*). *Developmental and Comparative Immunology,* 34, 1041-1050.

72. Zakrzewska, A., Cui, C., Stockhammer, O.W., Benard, E.L., Spaink, H.P., Meijer, A.H., 2010. Macrophage-specific gene functions in Spi1-directed innate immunity. *Blood,* 116, 1-11.

Table 1: MH Class IIβ genotype frequencies among the six families of *Oncorhynchus mykiss.* A total of 19 individuals were sequenced for families 4 and 9, meanwhile 18 individuals 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	0.17	-	-	-	-	-
DAB*1201/DAB*0801	0.17	-	-	-	-	-
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	-

Table 2: Genotype prevalence in mortalities and survivors to *F. psychrophilum* infection. A G-Test was performed on this contingency table using SPSS statistical software and the result is shown below.

Genotype	Percent of Mortalities	Percent of Survivors
DAB*1001/DAB*1001	32	40
DAB*1001/DAB*1101	18	13
DAB*0801/DAB*0801	3	2
DAB*1001/DAB*0801	19	31
DAB*1101/DAB*0801	6	2
DAB*1201/DAB*1001	3	2
DAB*1201/DAB*0801	5	2
DAB*0401/DAB*1001	2	0
DAB*0401/DAB*0801	2	0
DAB*0801/1001/1101	6	4
DAB*0404/0801/1001	3	0
DAB*1001/1101/1201	0	2
DAB*0401/0801/1001/1101	0	2

Figure Legends:

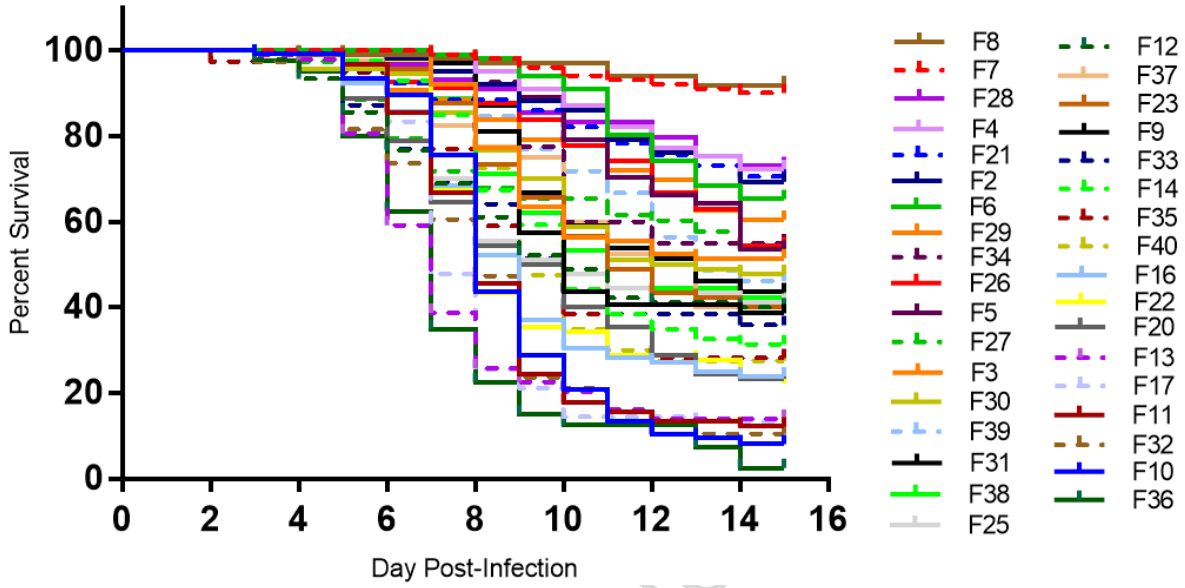
Figure 1: Survival curves throughout *F. psychrophilum* infection for the forty rainbow trout families analyzed for resistance/susceptibility to BCWD. **Panel A:** Survival curves for all rainbow trout families analyzed. **Panel B:** Survival curves for the six families used for MH class II β genotyping. **Panel C:** The survival curves for the eight families selected for the functional immune trials (Trials 1 – 3).

Figure 2: Indirect ELISA assay to compare serum IgM development against *F. psychrophilum* between resistant and susceptible families throughout infection. **Panel A:** Indirect ELISA results for serum IgM levels developed against *F. psychrophilum* between two resistant (F21 and F30) and two susceptible (F11 and F17) rainbow trout families throughout a 28-day infection trial. **Panel B:** Indirect ELISA results for serum IgM levels developed against *F. psychrophilum* between one resistant (F39) and one susceptible (F32) rainbow trout families throughout a 140-day infection trial. Serum from saline injected controls was also tested for antibody development to *F. psychrophilum* throughout the infection trial. **Panel C:** Survival curve for the two resistant (F21 and F30) and two susceptible (F11 and F17) families during the 28-day infection trial. **Panel D:** Survival curve for the resistant (39) and the susceptible (F32) families during the 140-day infection trial. Survival is only shown up to day 90 as there was no further mortality observed in this trial after this time. **Panel E-F:** Comparison of the observed mortality during the preliminary trial to determine resistance/susceptibility and the trials used to measure serum IgM against *F. psychrophilum*. Error bars represent the standard error of the mean (SEM).

Figure 3: Respiratory burst activity of HKL and total blood in resistant and susceptible families of rainbow trout. **Panel A:** RBA of HKL in one resistant (F39) and one susceptible (F32) rainbow trout family throughout *F. psychrophilum* infection. Control fish received i.p. injections of sterile CB. **Panel B:** RBA of HKL in another

resistant (F38) and susceptible (F36) rainbow trout family throughout *F. psychrophilum* infection. **Panel C:** Impact of *F. psychrophilum* on the RBA of total blood in a resistant (F38) and a susceptible (F36) rainbow trout family throughout *F. psychrophilum* infection. **Panel D:** Comparison of the observed mortality during the preliminary trial to determine resistance/susceptibility and the observed mortality in the trials used to measure RBA throughout *F. psychrophilum* infection. Error bars represent the SEM.

A



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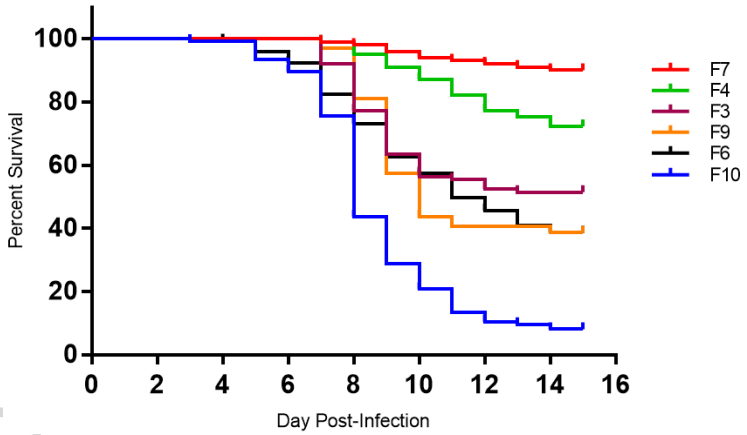

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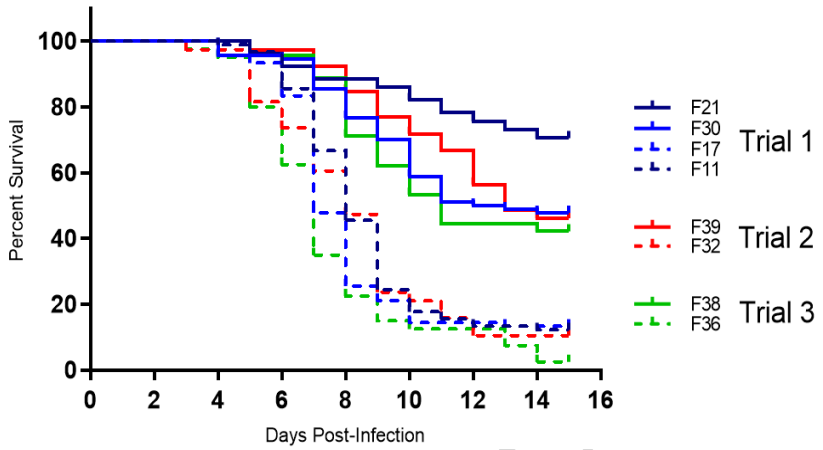

Figure 1

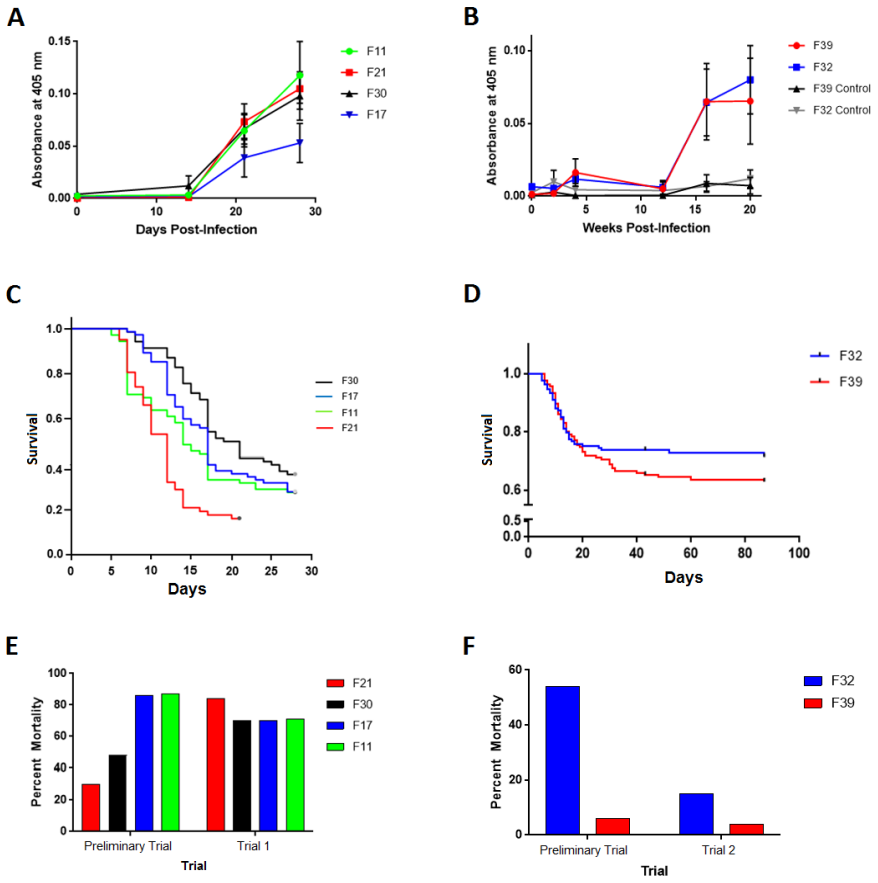

Figure 2

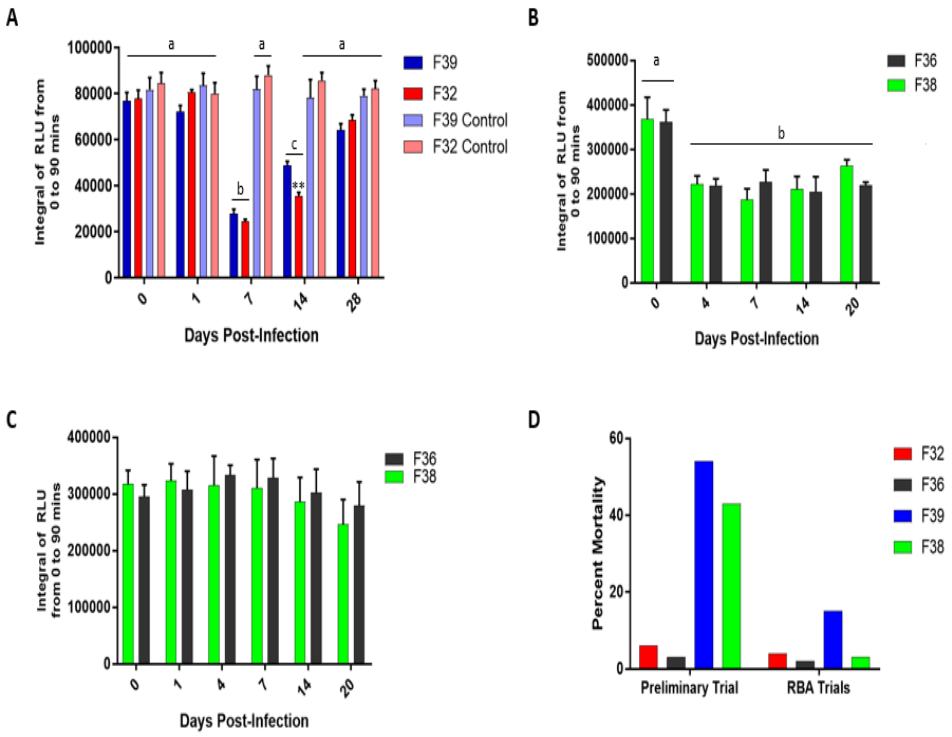

Figure 3

Highlights:

- Forty full-sibling rainbow trout families were created and resistance/susceptibility to *F. psychrophilum* infection was determined using experimental infection.
- Specific MH class IIβ amino acid sequences did not correlate with resistance/susceptibility.
- Serum antibody levels against *F. psychrophilum* did not differ between resistant and susceptible families.
- Respiratory burst activity of head kidney leukocytes dramatically decreased on Days 3-7 of infection (the days when mortality and morbidity was first observed) in both sensitive and resistant families.