F. psychrophilum resistant and susceptible rainbow trout show differences in abundance of IgT+ and IgM+ B cells

Erin Hennessey
College of William and Mary

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F. psychrophilum resistant and susceptible rainbow trout show differences in abundance of IgT+ and IgM+ B cells

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Thesis presented to the Undergraduate Faculty of The College of William & Mary in candidacy for the degree of Bachelor of Science

Biology Department

The College of William & Mary
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A thesis submitted in partial fulfillment of the requirements for the degree of Bachelor of Science in Biology

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May 3rd, 2017
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Abstract

Rainbow trout are heavily affected by *Flavobacterium psychrophilum*, a bacterium which is highly contagious in cold water. This bacterium causes Bacterial Cold Water Disease (BCWD) in the fish, which leads to severe symptoms and often results in death. This bacterium’s contagion poses a problem for trout hatcheries, which harvest over 1,000,000 lbs of these fish a year. The National Center for Cool and Cold Water Aquaculture has bred two distinct lines of rainbow trout: one line that is heavily susceptible to *F. psychrophilum*, and one that is resistant to it. Although this lab was able to genetically select the lines, the mechanisms which allow for resistance to *F. psychrophilum* is still unknown. We are using these two lines of fish to study differences in immune cell patterns between the lines.

This study focuses on IgM and IgT expressing (or secreting) B cells. Fish from each line were challenged with either phosphate buffer saline (PBS) or live *F. psychrophilum*. Using flow cytometric analysis and qPCR, B cell abundances were compared between lines and treatments. This study found that challenged susceptible fish show significantly less IgT+ B cells than resistant fish through flow cytometric analysis. Similarly, susceptible fish had significantly less tau immunoglobulin secreting cells than mock susceptible fish, mock resistant fish, and challenged susceptible fish. Additionally, significant negative correlations were seen between IgM+ or IgT+ B cells and pathogen load. Other significant negative correlations were observed between IgM or IgT secreting cells and pathogen load. These results lead us to believe that the IgT isotype is protective for rainbow trout, particularly in regards to this bacterium.
Chapter 1: Introduction

1.1 - The problem

Rainbow trout (Oncorhynchus mykiss) are a species of freshwater fish which are commercially farmed in hatcheries worldwide (Walbaum, 2005). The industry farms over 600,000 tons of fish yearly, bringing in a profit of about $2.6 billion (Walbaum, 2005). Flavobacterium psychrophilum (Fp) is a gram-negative bacterium known to cause Bacterial Cold Water Disease (BCWD) in rainbow trout. Symptoms of this disease include skin discoloration and lesions, corkscrewed swimming, loss of appetite, and eventually death (fig. 1) (Starliper, 2010). This bacterium also can create a biofilm around itself, making it sustainable without a host for long periods of time (Sundell et al., 2011). Consequently, the disease is quite contagious amongst rainbow trout. Because this bacterium is responsible for economic losses within hatcheries as a result of its viability, investigation into treatments of the disease is important to the industry.

The National Center for Cool and Cold Water Aquaculture (NCCCWA) has created two genetically selected lines of rainbow trout: one line that is resistant to F. psychrophilum, designated ARS-FpR, and a control line that is susceptible to F. psychrophilum, designated ARS-FpS (Wiens et al., 2013a). Although the mechanism for resistance is unknown, several studies have uncovered differences between the lines. After Fp challenge by intraperitoneal injection, resistant fish show significantly lower pathogen loads than their susceptible counterparts (Hadidi et al., 2008). Additionally, resistant fish have a higher survival rate 5-10 days after infection than do susceptible fish (Wiens et al., 2013a). These observations suggest a higher capacity for Fp control in ARS-FpR fish than in ARS-FpS fish.
1.2 - The immune system

The immune organs of rainbow trout differ from those of mammals, but both mammals and fish have both acquired and innate immunity. In mammalian species, acquired immunity is made up of B cells, T cells, and other pathogen-presenting cells, while innate immunity is comprised of leukocytes (natural killer cells, mast cells, eosinophils, and basophils) and phagocytic cells (macrophages, neutrophils, and dendritic cells) (Kindt et al., 2007). These different cells work in different ways. B cells secrete antibodies, which bind to a pathogen and help the immune system to detect and destroy it. Cytotoxic T cells secrete cytotoxic substances after recognition of an infected cell; helper T cells secrete cytokines to recruit other immune cells to a site of infection. Some leukocytes and phagocytic cells engulf antigens and digest them. Others help to recruit other immune cells to specific sites where pathogens are present. Regardless of function, all immune cells help to fight infection through inflammation – a localized immune response triggered by damaged tissue or entry of pathogens. Hematopoietic stem cells undergo maturation and differentiation in primary immune organs. In other words, these organs, which include the thymus and, more extensively, the bone marrow, are where immune cells are created. The actual immune responses occur within secondary immune organs. These sites include the spleen, lymph nodes, lymphoid tissue, tonsils, and localized areas of the gastrointestinal tract. Tertiary immune tissues also exist and are temporary sites formed during prolonged infection.

The immune organs of teleost fish differ from those of mammals. The anterior kidney (AK), also referred to as the head kidney, is the primary immune organ in fish. This is the site of hematopoiesis. Fish do not have bone marrow, which is the largest primary mammalian immune organ. The AK, therefore, is where B cells and myeloid cells are made in these fish. The secondary organs of these fish include the spleen (SPL), peritoneal cavity (PCF – peritoneal cavity fluid), and the blood (PBL – peripheral blood leukocytes). It is in these tissues where an antigen may encounter an immune cell and incite an inflammatory response in the body. The spleen contains mature B cells and short-lived plasma cells.
(SLPCs). When mature B cells are activated in the spleen, they differentiate into proliferating plasmablasts. Plasmablasts can then travel back to the AK and further differentiate into long lived plasma cells (LLPCs) for long term survival, or SLPCs which help to fight infection within the SPL. Plasmablasts and plasma cells secrete antibodies, which bind to pathogens to make them easily identifiable for digestion by the body. The peritoneal cavity is an area which encloses and protects various organs within the fish. This tissue also helps to store immune cells in a fish body. Lastly, blood is used by immune cells for travel to different tissues, but may also be a site where a B cell becomes activated. Because of this, immune cells are often found in the blood, and it is considered a secondary immune tissue.

Fish, like mammals, have both an acquired and innate immune system. In this paper, I will be focusing on the acquired immune system of rainbow trout, and more specifically, B lymphocytes.

1.3 - B cell development

B cells are formed from hematopoietic stem cells (fig. 2) (Kindt et al., 2007). When designated for B cell lineage through environmental factors, the hematopoietic stem cell will first differentiate into a lymphoid progenitor cell. After it is determined through transcription factors that this cell will become a B cell, it differentiates into a progenitor B cell. This cell will further differentiate into a pre B cell, and then

---

Figure 2 – B cell development. In the AK, hematopoietic stem cells give rise to developing B cells. Immature B cells travel to the SPL, where they differentiate into mature B cells which express different isoforms of immunoglobulin. When the immunoglobulin binds to a pathogen, the B cells activate to become ISC, which secrete immunoglobulin in the form of antibodies. LLPCs will travel back to the AK to fight infection there.
eventually into an immature B cell, at which stage an immunoglobulin (Ig) will appear. After an immature B cell is formed and is expressing an Ig gene, it travels through the blood to the spleen for storage or to help with an infection. In the blood or spleen, the immature B cell differentiates into a mature B cell, the state in which it will remain until it encounters a pathogen.

1.4 - B cell activation

If a pathogen binds to a mature B cell’s Ig, it activates, becoming an appropriately-named activated B cell (Kindt et al., 2007). It is thought that activated B cells start producing relatively small amounts of secreted Ig in the form of antibodies, in addition to expressing membrane-bound Ig (Barr et al., 2011). These B cells will further differentiate into plasmablasts, which are highly proliferative cells which produce more secreted Ig. Plasmablasts can then differentiate even further into an LLPC or SLPC. Plasma cells secrete the highest amounts of Ig compared to plasmablasts and activated B cells. Secreted antibodies will be able to selectively bind to the pathogen of interest and tag it for destruction. When antibodies bind to a pathogen, they create a complex which can be recognized by other elements in the immune system for degradation, as described below.

1.5 - Immunoglobulins

Immunoglobulins are proteins which are either bound to the membrane of B cells or secreted in the form of antibodies (Kindt et al., 2007). These proteins contain a specific binding site for pathogens, called the antigen-binding site. When antibodies bind to a pathogen, they create a complex which can be recognized by complement, a protein involved in the immune response. Complement can bind to the C terminus of an immunoglobulin. This antigen-antibody-
complement structure can then bind to complement receptors on phagocytic cells, which will then phagocytose the complex, eliminating the pathogen through digestion with cytotoxic substances which exist inside of the cell. Phagocytic cells can also recognize an antigen-antibody complex without complement. The C terminus of antibodies can be recognized by Fc receptors, which also exist on many phagocytic cells, like dendritic cells, macrophages, and neutrophils. The cells can then phagocytose the complex and degrade it, removing the pathogen. These two systems of antigen-antibody recognition help to keep the body healthy.

Immunoglobulins are composed of two different components: a heavy chain (HC) and a light chain (LC) (Kindt et al., 2007). Each HC and LC can also be broken down into two parts: the variable region and the constant region. The variable region of each chain contains tremendous diversity so that the immunoglobulin can bind to a wide array of antigens. This diversity in the variable region is created through segment rearrangement within the genes for these protein regions. The variable region in the heavy chain is composed of three segments: the V segment, the D segment, and the J segment (fig. 3). For each segment, one of multiple pieces may be randomly chosen to be incorporated as that particular segment. In the variable region in the light chain, two of these segments serve the same purpose: a V segment and a J segment. The heavy and light chain variable region combine to create an antigen binding site on the antibody. Many potential proteins can arise from the differential rearrangements of each strand, leading to an incredible variety in binding site specificity. Heavy and light chains also have variation in their constant regions. A similar rearrangement process occurs, in which a specific isotype is chosen. For the sake of assortment, different isotypes of constant region exist. Both of these different isotypes, also called classes, of immunoglobulin are localized to different tissues and may serve different functions. In rainbow trout, three isotype classes of immunoglobulin are observed within the heavy chain: immunoglobulin mu (IgM), immunoglobulin tau (IgT) (Hansen et al., 2005), and immunoglobulin delta (IgD) (Wilson et al., 1997). In my project, I will be focusing specifically on IgM and
The IgM isotype makes up most immunoglobulin in teleost tissues. It is essential for both systemic and mucosal responses (Salinas et al., 2011). Because of its prevalence, much of the literature available on teleost immunity focuses on IgM. IgT was discovered recently and is thought to have similar function to that of immunoglobulin alpha (IgA) in mammals (Hansen et al., 2005). IgT is localized in mucosal membranes, such as the gastrointestinal tract and the gills, but is also found in systemic tissues. Additionally, Ig can either be membrane bound or secreted in the form of antibodies. Ig shifts from membrane bound to secreted after a B cell is activated by a pathogen. In this project, I will be looking at B cells with membrane-bound IgM and IgT and at the expression levels of secreted IgM and IgT in resistant and susceptible fish. These expression levels will determine the relative amounts of plasma cells in each fish.

Three types of IgT have been observed: IgT1, IgT2, and IgT3 (Zhang et al., 2017). These different IgTs are classified by their expression levels within different tissues. IgT1 is found expressed highest in the blood and spleen, followed by the anterior kidney and gut. IgT2 is thought to be largely systemic, with almost all expression found in the thymus and blood. IgT3 is somewhat more elusive than its counterparts, having only been detected in the serum thus far. In this paper, I will be looking at all three types of IgT.

**1.6 - Research questions**

My first research question is: do resistant and susceptible fish show differences in B cell abundance in the blood and anterior kidney? I hypothesize that R-line fish will show a higher abundance of IgT+ B cells than S-line fish. Because IgT+ B cells are thought to be protective, R-line fish will show more, because these fish typically have low pathogen loads. Because S-line fish have high pathogen loads and lack protection, they will show a lower abundance of IgT+ B cells. Additionally, a previous study shows that naïve R-line fish have lower abundances of IgM+ B cells than naïve S-line fish (Zwollo et al., 2015). We would expect to see a similar pattern in this research. Another study shows that R-line fish
have larger spleens than S-line fish (Wiens, et al. 2013b). Although the significance of this remains unclear, it is possible that R-line fish are holding more immune cells in their spleens, like activated B cells and plasma cells, making them heavier. Because of this indication, I would expect to see large abundances of B cells in the spleen of R-line fish.

My second research question is: does Fp load affect the abundance of B cells in these immune organs? I hypothesize that fish with lower Fp load will show a higher abundance of B cells. If Fp load is low in a fish, this suggests that the fish is healthy and able to fight off the pathogen. If a fish is able to fight off a pathogen, it should have high abundances of functional and protective B cells. If a fish cannot fight off the pathogen, and therefore shows a high Fp load, it is likely that its B cells are either nonfunctional, in the wrong location, or missing entirely.

I will be using flow cytometric analysis to determine the abundance of IgM and IgT expressing B cells in the blood and anterior kidney. To more specifically look at secreted IgM and IgT (and therefore plasma cell abundance), I will be using qPCR.
Chapter 2: Materials and Methods

Animals and facilities

The rainbow trout used in this study were bred at the National Center for Cool and Cold Water Aquaculture (NCCCWA) in Leetown, West Virginia. They were reared following the NCCCWA Standard Operating Procedures for the Care and Use of Research Animals (Rainbow Trout). Initially, the fish were naïve with respect to Fp challenge and were maintained in a pathogen-free facility, which was monitored by inspections checking for bacterial and viral pathogens; these tests were negative for Flavobacterium psychrophilum (Wiens et al, 2013a). The fish remained at the NCCCWA until the post-hatch age of 4-6 months, at which time they were moved to The College of William & Mary Trout Facility. This facility contains 100-gallon tanks with a recirculating system using biologically-filtered water at 12° Celsius. IACUC committees at both the NCCCWA and The College of William & Mary approved this study (NCCCWA Protocol #076 and William & Mary protocol 2012-06-14-8016pxzwol). Steps were taken to reduce suffering within the fish. Trout used here were spawned in either year class 2013 (YC13) or year class 2015 (YC15). YC13 fish were challenged in 2014 (Fp2014 challenge experiment) and YC15 were challenged in 2016 (Fp16 challenge experiment).

In vivo Fp challenge

For the Fp2014 challenge, fish weight averaged 426 grams, and for the Fp2016 experiment, the average weight was 113 grams. On day 0 of the Fp2016 challenge, fish were intramuscularly injected near the dorsal fin with either 0.1mL sterile phosphate buffer saline.
(PBS) or freshly grown Fp CSF259-93 (fig. 4). The *F. psychrophilum* was cultured in TYES (4.4E7 cfu/fish, as determined by plate count) and resuspended in sterile PBS before the challenge. Collection occurred on day 5. One S-line fish died prior to collection. Fish were anesthetized, and blood and peritoneal cavity fluid (PCF) were collected in heparinized tubes. Cells collected from the PCF were placed in RPMI containing heparin. The fish were then euthanized, and spleen and anterior kidney were collected from each fish and placed in 5 mLs of ice cold RPMI. Small tissue samples of spleen and anterior kidney were collected from each fish, put into RNALater, and stored at -20 degrees Celsius to be analyzed later via qPCR. Red blood cells were removed using Histopaque-1077 (Sigma-Aldrich). Live cells were counted on a haemocytometer using Trypan blue. For blood cells, 25% of each sample was separately spun, resuspended into 1mL RNAzol, and stored at -20° Celsius to be analyzed later via qPCR. The remaining cells of the blood, spleen, peritoneal cavity, and anterior kidney were fixed for flow cytometric analysis.

**Antibodies**

The monoclonal mouse anti-trout immunoglobulin heavy chain mu (HCmu or I-14; DeLuca, 1983) was a gift from Dr. Greg Warr. The monoclonal mouse anti-trout immunoglobulin tau antibody (41.8; Zhang et al., 2010) was a gift from Dr. Oriol Sunyer. This antibody recognizes IgT1, IgT2, and IgT3 (Zhang et al., 2017).

**Fixation and permeabilization of cells for flow cytometry**

The cells were fixed in 1% ice cold paraformaldehyde (10% stock, EM-grade; Electron Microscopy Sciences). There were permeabilized in 1 mL of ice-cold methanol. The cells were incubated overnight at -20 degrees Celsius and then were either resuspended in permeabilizing solution (BD perm wash in PBS, BD Biosciences) and stained, or refixed and stored in FBS containing 10% DMSO (Zwollo et al., 2010). Cells remained stable in 96-well polystyrene round bottom plates (Fisher) at ~20 °C until needed for flow cytometric analysis.
Flow cytometry

Cells were removed from −20 °C and 1 mL of ice-cold PBS-SA was added. Cells were washed and pelleted twice in PBS-SA. After the second wash, the cells were resuspended in 1 mL of permeabilizing solution (BD perm wash, BD-Biosciences) containing 2% FBS. Cell pellets were resuspended in perm wash+5% FBS to 10x7 cells/mL and incubated for 15 min on ice with gentle shaking. Fluorescent Abs were freshly diluted to 10× Ab solutions (0.15 mg/mL) in perm wash containing 5% FBS. The 5 μL of 10× Ab solution was then added to 45 μL of cell suspension for a final antibody concentration of 0.015 mg/mL. Cells were incubated with the conjugated antibodies at 4 °C for 90 min in the dark with shaking. After the Ab incubation, 1 mL of perm wash+2% FBS was added to each tube and cells incubated in the dark for 10 min, shaking, at 4 °C. Cells were pelleted and resuspended in 1 mL of perm wash+2% FBS, incubated for 10 min with shaking, and spun as before. All supernatant was removed, cells resuspended in 100 μL perm wash containing 2% FCS. A maximum of 30,000 cells were acquired per well using a BD FACSArray (BD Biosciences), unless noted otherwise. Duplicate samples were analyzed for each experiment. Contour graphs were generated and shown as log algorithms with 50% intervals using WinMDI 2-8 software (J. Trotter 1993-1998).

DNA extraction

The frozen tissue samples were thawed, and about 50 mg of tissue was put into a 2 mL lysing tube containing 1 mL of DNAzol. Spleen samples required Lysing Matrix F tubes with beads; anterior kidney did not require beads in their lysing tubes. The tissue samples in lysing tubes were homogenized using an Omni beadruptor 24 (Omni International) at speed 5 for one 20-second cycle. 400 μL of UltraPure DNase/RNase-Free Distilled Water (Invitrogen, Inc.) was added to the tubes, and the DNA was purified according to manufacturer’s protocol. The concentration of DNA per sample was determined through a NanoDrop One Spectrophotometer (Thermo Scientific), and the samples were stored at −80° Celsius for future use.
RNA extraction and cDNA synthesis

The frozen tissue samples, which were stored in RNALater, were thawed, and around 50 mg of tissue were put into a 2 mL lysing tube containing 1 mL of RNAzol RT. Spleen samples required Lysing Matrix F tubes with beads; anterior kidney did not require beads in their lysing tubes. The tissue samples in lysing tubes were homogenized using the Omni beadruptor at speed 5 for one 20-second cycle. 400 μL of UltraPure DNase/RNase-Free Distilled Water was added to the tubes, and the RNA was purified according to the manufacturer’s protocol. The concentration of RNA per sample was determined through a NanoDrop One Spectrophotometer, and the samples were stored at -80 degrees Celsius for future use.

cDNA was created using iScript Reverse Transcriptase Supermix for RT-qPCR (Bio-Rad Laboratories, Inc.)

Quantitative real-time PCR

This technique was used to see expression of secreted heavy chain mu (secHCmu) and secreted heavy chain tau (secHCtau); the protocol used was from Schouten et al. (2013). For secHCmu expression levels, the primers amplify both IgM1 and IgM2 constant region genes. The expression of secHCtau1 was determined through use of a Taqman assay using a custom Taqman Gene Expression Assay (Applied Biosystems) with a FAM reporter, NFQ quencher, and a ROX reference. The sequence for the forward primer was 5’-CGGGTAACTCATGTGAAGACAAGT-3’. The reverse primer sequence was 5’-AGTCAATAAGAAGACACAACGACA-3’. The reporter sequence used was 5’-CACACAGGTAAAAATC-3’.

Trout α-tubulin (Tubulin 60) was used as the endogenous control (Schoute n et al., 2013). The Taqman assay for Fp loads was used (Marancik and Wiens, 2013). All assays were performed and observed using a StepOne Real-Time PCS instrument (Applied Biosystems).

A standard was ordered through gBlocks Gene Fragments (Integrated DNA Technologies, Inc.) which contained the desired number of DNA amplicons for the Fp assays. 500 ng of this standard was redistributed in 500 μL of TE buffer (10 mM Tris pH 8, 1 mM EDTA pH 8; 1 ng/μL). The dilutions were
created by mixing molecular grade water with samples to create standards of various concentrations of stocks between 10 and 1,000,000 copy/µL.

**Statistical Analysis**

Gated contours were used to determine population percentages from flow cytometry. For anterior kidney cells stained with HCmu and IgT, regions were created instead of a gate. This allowed for clearer determination of single and double positive stains. Percentages were taken from each region.

Data from secHCmu and secHCtau were reported as average CT scores from samples of three. Relative fold change for secHCmu was determined by subtracting the Δ [CT(secHCmu_ref)-CT(tubulin_ref)] of a control fish from the Δ [CT(secHCmu sample)-CT(tubulin sample)] of each sample to obtain ΔΔCT, and 2^ΔΔCT calculated for each sample. Similarly, for the RFC of secHCtau1, Δ [CT(secHCmu_ref)-CT(tubulin_ref)] of the same control fish was subtracted from the Δ [CT(secHCtauN_sample)-CT(tubulin_sample)] of each sample to obtain ΔΔCT, and 2^ΔΔCT calculated.

Data from Fp loads were taken as copy numbers of the target amplicon through the use of standard quantitation. The statistical programming environment R was used to calculate p values, create strip charts, and create scatterplots (R Core Team, 2015). Data comparing IgM+ and IgT+ B cells against Fp loads were log transformed using the package “dplyr” (Wickham et al., 2016). Data comparing challenged and unchallenged strains were checked for normal distribution using the Shapiro-Wilk normality test. Once normality was approved, ANOVA tests were run to check for significance between groups. After that, a Tukey-Kramer multiple comparisons of means test was performed using the package “multcomp” (Hothorn et al., 2016). Non-normally distributed data was log transformed and run through a Shapiro-Wilk normality test once again. If these still did not meet normality assumptions, a Kruskal-Wallis test was performed using the package “pgirmess” (Giraudoux, 2016). A nonparametric version multiple comparisons of means test, referred to as a Nemenyi test, was performed through the package
“PMCMR” (Pohlert, 2016). The Nemenyi test was set to use a Tukey distribution approximation for the sake of consistency with the parametric tests.
Chapter 3: Results

The two main research questions were as follows: Do naive R-line fish differ from S-line fish in their abundance of B cells, and do those patterns differ after in vivo challenge with Fp? Our approach involved intra muscular injection of live Fp, followed by euthanizing the animals and tissue collections on day 5 (Fp2016) or day 6 (Fp2014) post-challenge. Mock challenges involved injecting fish with PBS instead of Fp. Flow cytometric and/or gene expression analyses were then performed on peripheral blood lymphocytes (PBL) and spleen (SPL) from the Fp2016 experiment, or anterior kidney (AK) from the Fp2014 experiment.

3.1 - F. psychrophilum load

<table>
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<th>Fish</th>
<th>Fp Load</th>
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<td>MR12</td>
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<td>MS13</td>
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<td>MR16</td>
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Table 1 – Pathogen loads. Fish type are listed with their corresponding Fp load. Units are DNA nanograms/microliter.

F. psychrophilum load, or Fp load, describes the amount of pathogen DNA sequence found in each individual fish. A fish with a high Fp load shows more Fp DNA in its tissues and is likely sicker than a fish with a lower Fp load.

In each fish, the Fp load was determined through qPCR. Each sample was measured in triplicate, and the average was taken to determine final Fp load. All mock fish were negative for F. psychrophilum, as predicted, since these fish were not injected with any pathogen (table 1). Most R-line challenged fish had some pathogen, although a few were no longer infected at the time of collection. All S-line fish had
some pathogen. Averages for the 2016 challenge experiment were 450.48 copies of Fp DNA per 50 ng/µL for R-line fish and 16205.96 copies of Fp DNA per 50 ng/µL for S-line fish. For the 2014 experiment, the R-line average was 45.81 copies of Fp DNA per 50 ng/µL, and the S-line average was 2660.36 copies of Fp DNA per 50 ng/µL. As predicted, S-line fish showed higher amounts of pathogen load in both experiments, suggesting that S-line fish are less capable of fighting the bacteria and therefore show more of them after 5 or 6 days. Standard errors were high, in some cases exceeding the average, because of the huge variation in copies of Fp DNA among the individual lines. The range for 2016 challenged R-line fish was 0 to 3021.52 copies of Fp DNA per ng/µL. The range for the 2016 challenged S-line fish was 0 to 63,100 copies per 50 ng/µL. The range for 2014 challenged R-line fish was 0 to 852.5 copies. The range for 2014 challenged S-line fish was 1.9 to 11,557.9 copies per 50 ng/µL.

3.2 - qPCR data shows amounts of secreted mu and tau transcripts in AK Fp2014

qPCR was used to look at expression levels of immunoglobulin in the form of fold change. The fold change values reflect average levels of expression for each secreted immunoglobulin: tau and mu. Because these antibodies are thought to play a role in protection for the fish, we hypothesized that R-line fish would show more secreted HCtau and HCmu transcripts than S-line fish.

α tubulin was used as an endogenous control to calculate the relative fold change of both secreted tau and HCmu. As mentioned above, these qPCR values are from the AK in the 2014 challenge experiment. After collection of relative fold changes using qPCR, both fold change and Fp values were log transformed after adding a constant of 1 to account for values which were 0. All values were put into R to be analyzed using ANOVA tests and Pearson product-moment correlation tests. Scatterplots and strip charts were made for visualization of a best fit line and equation.
3.2.1 — Expression levels of secreted tau and mu between MR, MS, FpR, and FpS – AK Fp2014

Relative fold changes of both secreted HClmu and HCltau were compared against both R- and S-lines for all treatment groups. Data were compared in R through either an ANOVA test (for normally distributed data) or Kruskal-Wallis test (for non-normally distributed data). This comparison showed significant differences in tau fold change between challenged S-line fish and both mock and challenged R-line fish, suggesting that challenged S-line fish have lower expression levels of tau than mock and challenged R-line fish (fig. 5A). However, no significant difference was seen between mock and challenged S-line fish, suggesting that secreted tau levels remain relatively constant in S-line fish in the AK after challenge with Fp. No significant difference was seen between any treatments for secreted HClmu expression (fig. 5B).

![Figure 5](image-url)  
*Figure 5 — Secreted HCmu and tau fold changes across treatments in AK Fp2014. Significance of secreted tau (A) and mu (B) expression levels between mock resistant (MR), mock susceptible (MS), challenged resistant (FpR), and challenged susceptible (FpS) fish was calculated using multiple comparison of means tests. Means are shown as thick horizontal bars, and standard error of the mean is shown extending from the mean. These data are from qPCR of AK Fp2014. *p < 0.05. **p < 0.01.*

3.2.2 — Correlation between secreted mu and Fp load – AK Fp2014

Secreted levels of HClmu were looked at in comparison to Fp loads in all fish in the AK from Fp2014 samples. Secreted HClmu in the AK showed no significant difference in either R-line, S-line, or
pooled challenge data, suggesting that levels of secreted HCMu expression may remain constant in this tissue, regardless of pathogen load (table 2).

### 3.2.3 — Correlation between secreted tau and Fp load — AK Fp2014

Secreted levels of tau were looked at in comparison to Fp loads in all fish from the Fp2014 AK samples. Secreted tau in the AK showed a negative correlation between secreted tau levels and Fp load in pooled challenge samples (p = 0.009, table 2). No correlation was observed between the individual lines and pathogen load. Scatterplots were created to visualize a line of best fit. These graphs incorporate the equation for the

<table>
<thead>
<tr>
<th>Pooled Fp-challenged Fish</th>
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<th>Secreted Tau</th>
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<th>Susceptible Fp-challenged Fish</th>
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<th>Secreted Tau</th>
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<td>PBL Day 5</td>
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Table 2 — Correlation between secreted antibodies and Fp load. Significance between secreted mu or tau and Fp load was calculated using a Pearson’s product-moment correlation test on Fp load values and expression levels taken from qPCR. Significance was calculated in all lines together, and then divided into R-line and S-line alone. AK Day 6 samples were from the Fp2014 experiment, and PBL Day 5 samples were from the Fp2016 experiment. Significance is shown in bold.

**Figure 6 — Tau expression levels against Fp load.** Scatterplots with a line of best fit is shown for pooled data (A), R-line data alone (B), and S-line data alone (C). The equation for line of best fit and R-squared values are shown within the graphs. These data are from qPCR of AK Day 6 Fp2014.
line of best fit for pooled, R-line, and S-line samples (fig. 6). Again, note that only the pooled samples show a significant correlation between secreted tau and Fp load (fig. 6A).

3.3 - qPCR data show amounts of secreted mu and tau transcripts in PBL Fp2016

Again, qPCR was used to look at expression levels of mRNA for antibodies in PBL. The fold change values reflect average levels of expression for each secreted immunoglobulin: tau and mu. Because these antibodies are thought to play a role in protection for the fish, we hypothesized that R-line fish would show more secreted tau and HCMu transcripts than S-line fish.

α tubulin was used as an endogenous control to calculate the relative fold change of both secreted tau and HCMu. These qPCR values are from the PBL in the 2016 challenge experiment. After collection of relative fold changes using qPCR, both fold change and Fp values were log transformed after adding a constant of 1 to account for values which were 0. All values were put into R to be analyzed using ANOVA tests and Pearson product-moment correlation tests. Scatterplots and strip charts were made for visualization of a best fit line and equation.

3.3.1 – Expression levels of secreted tau and mu between MR, MS, FpR, and FpS – PBL Fp2016

Figure 7 – Secreted mu and tau fold changes across treatments in PBL Fp2016. Significance of secreted tau (A) and mu (B) expression levels between mock resistant (MR), mock susceptible (MS), challenged resistant (FpR), and challenged susceptible (FpS) fish was calculated using multiple comparison of means tests. Means are shown as thick horizontal bars, and standard error of the mean is shown extending from the mean. These data are from qPCR of PBL Fp2016. *p < 0.05. **p < 0.01.
Relative fold changes of both secreted HCmu and tau were compared against both R- and S-lines for all treatment groups. Data were compared in R through either an ANOVA test (for normally distributed data) or Kruskal-Wallis test (for non-normally distributed data). This comparison shows significant differences in tau fold change between challenged S-line fish and both mock and challenged R-line fish, suggesting that challenged S-line fish have significantly lower expression of tau than mock and challenged R-line fish (fig. 7A). HCmu fold change was also significant between challenged S-line fish and both mock and challenged R-line fish, suggesting that challenged S-line fish have significantly lower expression of HCmu compared to mock and challenged R-line fish (fig. 7B). No significant difference was seen between mock and challenged S-line fish for both tau and HCmu expression, the data was close (p = 0.07 for both tau and HCmu). This lack of significance may suggest that secreted tau and HCmu levels remain relatively constant in S-line fish in the PBL after challenge with Fp.

3.3.2 — Correlation between secreted mu and Fp load – PBL 2016

Secreted levels of HCmu were looked at in comparison to Fp loads in all fish in the PBL. Secreted HCmu in the PBL only showed a negative correlation between secreted HCmu and Fp load in the PBL in the pooled data (p = 0.033, table 2). No significant correlation was observed for S- or R-line fish alone. The S-line fish alone were not significant, with a p-value of 0.08 and an N value of 6. It may be important to note that the R-line fish alone had an N value of 3. Both R- and S-line fish alone have relatively small N values and may benefit from further sampling to draw more convincing conclusions.

3.3.3 — Correlation between secreted tau and Fp load – PBL Fp 2016

Secreted levels of tau were looked at in comparison to Fp load in all fish in the PBL. Secreted tau in the PBL showed a negative correlation between secreted tau and Fp load in pooled samples (p = 0.041, table 2). R-line fish alone also showed a negative correlation between secreted tau and Fp load in the R-line alone (p = 0.016, table 2). S-line fish alone showed no significant correlation and had an N value of 7.
Again, both R- and S-line data for tau relative fold change have N values below 10, and would benefit from further sampling to draw more convincing conclusions.

3.4 - Flow cytometry – SPL Fp2016

Flow cytometry was used to look at the abundance of B cells expressing IgT and IgM in all fish in the SPL. Because these B cells are thought to be protective for the fish, we expected to see significantly larger abundances in the R-line fish than in the S-line fish. The abundance of cells was measured by taking percentages of positively staining cells from the quadrants of each contour graph. The data were put into R to be analyzed through a Pearson’s product-moment correlation test and graphed in scatterplots.

3.4.1 – Comparing abundance of IgM+ and IgT+ B cells in MR, MS, FpR, and FpS – SPL Fp2016

Abundance of IgM+ and IgT+ B cells were compared against both R- and S-line fish for all treatment groups in the SPL. Data were compared in R using either an ANOVA test or Kruskal-Wallis test. Abundance of IgM+ B cells showed no significant difference between treatment groups (fig. 8A). Abundance of IgT+ B cells were significantly different in mock R-line fish than in challenge R-line fish,

![Figure 8](image-url)
mock S-line fish, and challenge S-line fish (fig. 8B). These data suggest that mock R-line fish have a significantly larger abundance of IgT+ B cells; however, the N value is 5. Further sampling may be necessary to draw more convincing conclusions.

3.4.2 — Correlation between IgM+ B cells, ISCs, and Fp load — SPL Fp2016

![Figure 9](image)

**Figure 9** — Challenged R- and S-line fish show populations of IgM+, IgM++, IgT+, and IgT++ B cells. Each of these contour graphs represents a single fish’s cellular composition. The mock fish are shown on the top (A), while the challenged fish are on the bottom (B). The R-line fish are on the left, and the S-line fish are on the right. The cell population in the blue gate shows IgM+ B cells, and the population in the yellow gate shows IgM ISCs. The population in the red gate shows IgT+ B cells, and the population in the green gate shows IgT ISCs. These data are from SPL Fp2016.

<table>
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<th>Stains</th>
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<th>Resistant Fp-challenged fish</th>
<th>Susceptible Fp-challenged fish</th>
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<tr>
<td>IgT++</td>
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<td>6</td>
</tr>
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**Table 3** — Correlation between flow cytometric stains and Fp load in SPL Fp2016. Significance between stains and Fp load was calculated using a Pearson’s product-moment correlation test on Fp load values and cell abundance taken from flow cytometry. Significance was calculated in all lines together, and then divided into R-line and S-line alone. All data are from SPL Fp2016. Significance is shown in bold.
In the SPL, cells were stained through flow cytometry using an IgM marker. Cells which stained positively for this marker suggest the presence of IgM on the cell surface. A population of IgM+ B cells was observed in contour graphs and is outlined in blue in figure 9. This population is likely mature B cells and activated B cells. IgM secreting cells, likely made up of plasmablasts and plasma cells, are outlined in yellow in figure 9.

In the pooled data of challenged fish, IgM+ shows a significant negative correlation between IgM+ B cells and Fp load in R- and S-line fish combined (table 3). This regression can be visualized in figure 10A. No significant correlation is seen in IgM+ B cells for R- or S-line fish alone, nor was there any significant correlation in ISCs.

**Figure 10 - %IgM+ B cells against Fp load.** Scatterplots with a line of best fit is shown for pooled data (A), R-line data alone (B), and S-line data alone (C). The equation for line of best fit and R-squared values are shown within the graphs. These data are from flow cytometry of SPL Fp2016.
In the SPL, cells were stained through flow cytometry using an IgT marker. Cells that stain positively for this marker suggest the presence of IgT on the cell surface. A population of IgT+ B cells was observed in contour graphs and is outlined in figure 9. This population is likely mature B cells and activated B cells. IgT+ secreting cells, likely plasmablasts and plasma cells, are outlined in green in figure 9.

IgT+ B cells show a significant negative correlation between IgT+ B cells and Fp load in R- and S-line fish combined (table 3). Figure 11 shows these data points with a regression. No significant difference is seen in IgT+ B cells for R- or S-line fish alone, nor is there any significant correlation seen in IgT secreting cells for all fish.
Chapter 4: Discussion

The research questions addressed here were: do R-line and S-line fish show differences in IgM+ and IgT+ B cell abundance, and are the patterns maintained after in vivo challenge with Fp? Because previous studies have shown that naïve R-line fish have lower abundances of IgM+ B cells than S-line fish, we expected to see similar results in our challenge study (Zwollo et al., 2015). Not only did we see a lower abundance of IgM+ B cells in R-line fish before challenge, but we also observed a higher abundance of IgT+ B cells in R-line fish than in S-line fish before challenge. Both of these patterns remained after challenge, as well. The results also show that IgT+ B cell abundance and secreted tau expression correlates with lower pathogen load in challenged fish.

4.1 - Secreted and membrane-bound IgT

4.1.1 – SPL

The SPL is a common site of immune response, where activation of mature B cells takes place. During infection, activated B cells differentiate into highly proliferative plasmablasts, which further differentiate into plasma cells. Both plasmablasts and plasma cells secrete immunoglobulin, which help to tag pathogens for degradation. In an uninfected SPL, we would expect to see primarily mature B cells, but no activated B cells or plasmablasts. In an infected SPL, we would expect to see more diversity in B cell types – activated B cells, plasmablasts, and plasma cells – because they are needed for secretion of pathogen-specific antibodies.

Here, we studied the B cell composition in the SPLs of resistant and susceptible trout, both before and after challenge with Fp. We observed that the unchallenged R-line fish had a significantly higher abundance of IgT+ B cells than challenged R-line fish, and both unchallenged and challenged S-line fish. This was determined through multiple comparison of means tests on percentages of IgT+ staining cells in flow cytometry.
Because the mock resistant fish are unchallenged, we would not expect to see any activation of their B cells, and for this reason, we expect these IgT+ cells to only be mature IgT+ B cells. These findings suggest that mock R-line fish have significantly more mature B cells in the SPL than all other treatments. It is possible that the mock R-line fish initially have more mature IgT+ B cells in the SPL than the mock S-line fish, offering an advantage when the fish are infected. With more mature IgT+ B cells, R-line fish may more rapidly or efficiently respond to pathogens than S-line fish.

Mock R-line fish were also found to have significantly more IgT+ B cells than challenged R- and S-line fish. When fish are challenged, their mature B cells (the cells we are observing in mock fish SPLs) are activated and differentiate into plasmablasts and plasma cells. The abundance of mature B cells would be reduced in fish that respond stronger to the Fp. This may account for the significant differences between mock R-line and challenged R- and S-line fish. Next, levels of secreted HCTau was also calculated using qPCR in PBL and AK.

4.1.2 - PBL and AK

In the PBL and AK, secreted tau levels were determined through qPCR. PBL, like the SPL, is a secondary immune tissue. PBL is used for the transportation of B cells to sites of infection, to sites of maturation, and is sometimes utilized as a site of maturation itself. Because of this, we would expect to see immature B cells, mature B cells, and plasma cells in the PBL. In an infected fish, we may also see activated B cells after encountering a pathogen in the PBL or traveling to another infection site, or we may see low levels in the PBL, as cells have moved into infected tissue. The AK is a primary immune organ in fish and is the site of hematopoiesis for B cells. The AK is also used as storage for LLPCs. In this tissue, we would expect to see immature B cells and LLPCs. Using qPCR, we are only focusing on cells which secrete Ig, which includes plasmablasts and plasma cells.

Secre ted levels of HCTau, and therefore abundance of plasmablasts and plasma cells, were determined through qPCR of PBL and AK tissues. Through this technique, it was found that challenged S-
line fish have significantly lower secreted tau levels than both mock and challenged R-line fish in both tissues. This has different indications for each tissue. In the AK, this suggests that challenged S-line fish have less IgT secreting cells than mock and challenged R-line fish. These cells are likely used to help fight infection, and since challenged S-line fish have less of them, they may be less equipped to respond to infection. Challenged S-line fish showed no significant differences of secreted HCTau when compared to mock S-line fish in the AK. This may indicate that mock S-line fish have similar amounts of LLPCs compared with challenged S-line fish, further suggesting that susceptible fish do not “lose” tau secreting cells upon infection, but begin with less altogether. More plausibly, our results were inconclusive because of a relatively low N value. Visually, it appears that mock S-line fish have similar secreted tau levels to both mock and challenged R-line fish, although no significance was calculated. Had this experiment been performed on hundreds of fish, a more concrete significance (or insignificance) may be seen between S-line fish treatments.

In the PBL, challenged S-line fish have significantly lower tau expression levels than challenged and mock R-line fish. This suggests that susceptible fish are not circulating as many tau secreting cells as resistant fish are. Because resistant fish show more of these plasma cells, it is likely that they offer protection to the fish. Again, challenged S-line fish show no difference from mock S-line fish, which may indicate that susceptible fish begin with less circulating tau secreting cells, but is likely a result of a small sample size.

4.2 - Secreted and membrane-bound IgM

4.2.1 – SPL

Abundance of IgM+ B cells was calculated in the SPL using contours from flow cytometry. R-line and S-line fish were observed before and after challenge. IgM+ staining cells in the SPL before challenge are likely mature B cells with membrane-bound IgM. After challenge, we may expect these cells to differentiate into activated B cells This technique does not include IgM ISCs, and therefore we cannot
make assumptions about plasmablasts and plasma cells. No significant differences were seen between challenged or unchallenged R- and S-line fish.

The amount of mature B cells expressing IgM in naïve fish is similar to the amount of activated B cells which appear in infected fish. This is likely because these cells do not leave the SPL when infected; they may stay in this tissue to help fight the pathogen. The lack of difference between R- and S-line cells expressing IgM suggests that perhaps IgM is not playing a significant role in protection. Because R-line fish do not show a larger abundance of IgM+ B cells, we may conclude that these are not the population which is contributing to their resistance to Fp.

4.2.2 - PBL and AK

Abundance of IgM ISCs were determined using qPCR in both the PBL and AK. In the AK, no significant difference was seen between treatments. In the PBL, challenged S-line fish have significantly less IgM ISCs than challenged and unchallenged R-line fish.

No significant difference between challenged and unchallenged R- and S-line fish in the AK suggests that the same amount of IgM ISCs are present in all AKs, regardless of treatment. ISCs in the AK may only include LLPCs, since other cells most abundant in the AK (like pre B cells and immature B cells) do not secrete immunoglobulin. This is in accordance with our SPL data, which also shows no significance between IgM ISCs between lines or treatments. It is possible that a constant amount of IgM ISCs are being created and transported back to the AK, because this isotype is not particularly protective.

In the PBL, however, challenged S-line fish show significantly less IgM ISCs than both challenged and unchallenged R-line fish. This indicates that fewer ISCs are being transported between tissues in challenged susceptible fish, although the amounts of ISCs in the tissues remain the same (as per SPL and AK data). PBL is not only used for travel between immune tissues, but also travel to specific sites of infection. It is possible that, although IgM ISCs remain the same in immune tissues, larger amounts of IgM ISCs are being transported to other places of the body. IgM is both systemic and mucosal, and can
potentially be found in many other tissues which were not observed in this study, like the gastrointestinal tract or the gills. If this were the case, then IgM may pose some protective benefits for R-line fish, since challenged S-line fish have significantly less.

When compared against Fp load, no significant difference in IgM ISC abundance is seen in the AK, but a significant negative correlation is seen in PBL. The lack of significance is consistent with our other findings for IgM ISCs in the AK – these data confirm that Fp challenge does not affect the abundance of IgM ISCs in the AK. In the PBL, fish with higher pathogen loads show fewer IgM ISCs. This is also consistent with our other data from the PBL. It is possible that, because IgM is both systemic and mucosal, ISCs are traveling in the PBL to other parts of the body from which we did not collect data. If this is the case, it is possible that IgM poses protective advantages, because a larger abundance is found in R-line fish than in challenged S-line fish.

4.3 – Conclusions

The data presented here supports my hypotheses that 1) R-line fish will have larger abundances of IgT+ B cells than S-line fish, and 2) that larger abundances of IgT+ and IgM+ B cells will be seen in fish with lower Fp loads. This experiment showed that R-line fish had significantly higher amounts of IgT+ B cells and ISCs, indicating that IgT is particularly protective for rainbow trout infected with *F. psychrophilum*.

Future studies should attempt to determine which genetic mechanisms support the expression and proliferation of IgT over IgM. This may help us to further understand why S-line fish show less IgT+ B cells and ISCs, and why they respond poorly to Fp. A pathway which favors IgT over IgM may be induced in S-line fish to help their immune system fight infection.

Additionally, the study of other immune cells in these fish, like T cell and dendritic cell abundance, may elucidate more on the mechanism of resistance. Initially, this study intended to also observe myeloid cell differences between the lines. It may be helpful to study T cells, which also play a
role in the acquired immune system. These cells may also show different patterns in different treatment
groups and fish lines.

Because IgM and IgT are both components of mucosal immunity, studying other tissues through a
similar challenge experiment, like the gills and gastrointestinal tract, may elucidate more patterns and
help to explain certain “losses” of B cells from challenged S-line fish PBL and SPL.

An experiment which tags membrane-bound IgM and IgT before challenge may illuminate to
where these cells migrate after challenge. In such an experiment, the migration patterns of membrane-
bound IgM and IgT can be compared in R- and S-line fish. This may also explicate other tissues which play
an important role in this immune response.

Lastly, and somewhat unrelated to my conclusions, a study on the mechanisms of the bacteria
may prove useful in the search to eliminate BCWD. As mentioned in the introduction, *F. psychrophilum*
has the ability to form a biofilm around itself, providing the ability to survive without a host for years. A
water treatment which lyses this biofilm (without hurting the fish) may decrease the bacteria’s contagion.
References


