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Quantitative Analysis of the Alternative Splicing of Pax5 in Teleost Fish During Spawning

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A Quantitative Analysis of the Alternative Splicing of Pax5 in Teleost Fish During Spawning

A thesis submitted in partial fulfillment of the requirement for the degree of Bachelor of Science in Biology from The College of William and Mary

by

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# Table of Contents

Abstract ................................................................. 1

I. Introduction .......................................................... 2

II. Methods .............................................................. 23

III. Results ............................................................... 32

IV. Discussion ........................................................... 53

Attachment 1 ............................................................ 60

Appendix ................................................................. 64

References ............................................................... 109
Abstract

Pax5 is the master regulator of B-cell development. Pax5 is highly conserved between invertebrate and vertebrate species and is known to be alternatively spliced. An isoform missing exon 2 appears to be species independent. This is significant because exon 2 contains the DNA binding domain and thus these isoforms have decreased DNA binding, which change their function as a transcription factor. This project aimed to determine if there are changes in the alternative splicing of Pax5 during a humoral immune response in spawning sockeye salmon (Onchorynchus nerka). Specifically, this project looked at the changes in Onchorynchus Pax5 isoforms that lack exon 2, to determine if these changes correlated with an increase in the amount of secreted IgM in such animals. Real-time PCR was performed using primers that amplified full length Pax5, the Pax5Δ2 isoform, and secreted and membrane IgM. The relative expression levels of Pax5Δ2/Pax5FL were then compared to the relative expression levels of secreted/membrane IgM. This project concluded that there is a positive correlation between the levels of secreted IgM and Pax5Δ2. This positive correlation was observed in vivo in O. mykiss in the laboratory, in vitro in LPS activated B-cells from O. mykiss, and in vivo in spawning O. nerka samples for spleen and blood. This conclusion supports the model that Pax5Δ2 is acting as a co-repressor of Pax5FL by binding to the initiation complex on the Xbp1 gene, thus changing the shape of the initiation complex and preventing Pax5FL from binding to the promoter region of the Xbp1 gene.
Introduction

The immune system is a highly adaptable system that has evolved to protect organisms from pathogens. To adequately protect against pathogens, the system must be able to both recognize and respond to foreign invaders. In vertebrates, there are two systems of immunity. Innate immunity is the highly effective first line of defense, which includes molecular and cellular mechanisms that are poised and ready to prevent and eliminate infection. In contrast, adaptive immunity develops in response to an infection and adapts to recognize, eliminate, and remember the foreign invader. Collaboration between the innate and adaptive immune responses increases the effectiveness of an immune response. This thesis focuses on the adaptive immune system in fish. The following briefly reviews the adaptive immune system in the well-characterized mammalian species, mouse and humans.

The Adaptive Immune System in Mammals.

The main players in adaptive immune responses are the lymphocytes. Lymphocytes are a type of white blood cell produced in the bone marrow. Upon leaving the bone marrow, lymphocytes circulate in the blood and lymphatic system and reside in various lymphoid organs. The two major populations of lymphocytes are B lymphocytes (B cells) and T lymphocytes (T cells). This work focuses on B cells. B cells are the main players in humoral immunity (B cell response), and produce antibody or immunoglobulin. This “humoral” immune response protects against extracellular
bacteria, viruses, and foreign macromolecules through the binding of immunoglobulin, present in the plasma, lymph and tissue fluids.

After maturing in the bone marrow, B cells express unique antigen-binding receptors on their membrane, known as “membrane-bound” immunoglobulins. When an antigen binds to “membrane-bound” immunoglobulins, it causes the cell to divide. The progeny differentiate into memory B cells and effector B cells (plasma cells). Memory B cells have a long life span and are prepared for another encounter with the same antigen. The majority of plasma cells only have a short half-life, but are able to produce very high quantities of a secreted form of antibody or immunoglobulin in that time. These secreted antibodies are the major effector molecules of humoral immunity (Kuby). A small population of so-called long-lived plasma cells will move to the bone marrow and may keep secreting antibodies for many years (Elliyard et al. 2004 and Bromage et al. 2004).

In mammalian species, immunoglobulins are composed of four peptide chains, two identical light chains, and two identical heavy chains. Mammalian species have five different heavy chain constant regions, \( \mu, \gamma, \epsilon, \alpha, \) and \( \delta \). The heavy chains of the immunoglobulin determine the class of the antibody: IgM (\( \mu \)), IgG (\( \gamma \)), IgA (\( \alpha \)), IgD (\( \delta \)), IgE (\( \epsilon \)). The five classes of immunoglobulin can be expressed either as secreted or membrane immunoglobulin. The carboxyl-terminal domain in secreted immunoglobulin is both structurally and functionally different from the corresponding domain in membrane-bound immunoglobulin. Immunoglobulins bind to antigen, which invokes a number of responses that result in the removal of the antigen and death of the pathogen. This honors research used the ratio of secreted/membrane IgM as a marker of immune response. The membrane bound \( \mu \) heavy chain is expressed on mature, resting B cells. In
contrast, plasma cells lack membrane IgM but make high levels of secreted IgM. Hence, the more activated a cell, the higher the ratio of secreted IgM/membrane IgM becomes.

**Figure 1:** A resting, mature B-cell upon activation, becomes activated, then becomes a plasmablast, and finally, a plasma cell. The amount of membrane IgM decreases during this process, while the amount of secreted IgM increases. (from Zwollo, 2011).

**The Teleost Model**

Our lab uses teleost fish as a model organism to study the immune system. It is important to study the teleost clade for a number of reasons. The appearance of the jaw in evolutionary history is coordinated with the development of an adaptive immune system, thus teleosts represent one of the earliest clades to have this system (Matsunaga and Rahman, 1998). The lymphocytes of teleost fish are highly diverse, similar to what is seen in humans. Thus, a study of the B-lymphocytes in teleost fish could bring us one-step closer to answering vital questions related to disease in human populations.

Additionally, the farming of teleost fish is a source of food worldwide. These populations of farmed fish are susceptible to a number of diseases. Basic prevention in the form of sanitation, chemical treatment of the water, and antibiotics in food are helping to limit the loss of fish currently. Studies on the immune system of fish could further the
development of both preventative measures and treatment options for these diseases, thus keeping the animals healthy (Food and Ag. Organization of the United Nations).

Previous studies have begun to dissect the immune system of teleost species, specifically the rainbow trout. Teleosts are an interesting model for immunology research because these organisms do not possess bone marrow or lymph nodes. In teleosts, the major immune organs are the kidney, spleen and blood. The anterior kidney is the main site of hematopoesis in the teleost (Zwollo et al., 2005, 2010, Zapata and Cooper, 1990, and Katarri and Irwin, 1985). Additionally, research has shown that there is a B-cell maturation gradient along the teleost kidney: the anterior kidney is home to (mostly proliferating) B cell precursors and long-lived plasma cells while the posterior kidney is home to partially activated B-cells as well as plasmablasts (Zwollo et al., 2005). The blood lacks plasma cells and contains mostly resting cells (Zwollo et al., 2005, 2008). In vitro lipopolysaccharide (LPS) induction of mature B-cells causes splenic cells to progress from plasmablast to the plasma cell stage, while B-cells in the blood do not generate plasma cells and generate much fewer plasmablasts. Thus, B-cell populations vary significantly between the blood, spleen, posterior kidney and anterior kidney. Based on these data, previous research has indicated the anterior kidney as a primary immune organ in teleosts while the posterior kidney and spleen function as secondary immune organs. Additionally, the blood has been characterized as a reservoir to store and transport B-cells. Figure 2 summarizes the types of B-cells found in each immune tissue in teleosts (Zwollo et al, 2005).

In land vertebrates, there are several Ig classes with differing structure and function produced by B-cells. In fish, immunoglobulins are of lower affinity and there
are only three classes of Ig, with the main type corresponding to mammalian IgM. IgM is a pentamer with ten binding sites in most vertebrate species, but in fish it is composed of four basic units with eight binding sites, the lack of two binding sites is thought to be responsible for less efficient Ig binding in teleost fish (Pilstrum and Bengten, 1996). In addition to IgM, teleost B-cells have very low percentage of IgT and IgD. Interestingly, in fish, there appears to only be one gene for the IgM, but the gene for the IgD and IgT has been duplicated, possibly due to the tetraploid ancestry of salmonoid fish (Hansen et al., 2005).

**Figure 2**: Model showing the stage of B cell development and activation cells in each of the immune organs of the teleost. (Zwollo et al. 2005). This model illustrates the maturation gradient. B cell activation occurs in the Posterior Kidney as well as the Spleen. According to this model, once cells have matured all the way to the plasma cell stage, they can travel back into the anterior kidney and survive as long-lived plasma cells.

Transcription factor structure and function is conserved between species due to the highly conserved nature of DNA binding domains. Therefore, using transcription factors as developmental markers in species such as teleosts, which are much less well-studied than mammalian species, allows researchers to take a comparative approach to the study of gene regulation. Trout Pax5 protein has 79% homology with mouse Pax5
indicating that it is functionally relatively similar to its mammalian homolog. More importantly, the paired domain, or DNA binding domain, of transcription factors is the most highly conserved domain of the Pax5 protein, with homology as high as 95-99% (Zwollo, 2011). The ability to use antibodies to mammalian homologs of the gene highlights the importance of this comparative approach (reviewed in Zwollo et al, 2011).

Pax5 is a Regulator of B-cell Development

Pax5 is a transcription factor found in vertebrate B cells and adult testis as well as in the midbrain of developing embryos. This research focused on the Pax5 gene in teleost B-cells. Studies in mammals have shown that the differentiation of hematopoietic stem cells (HSC) into B-cells is a process that must be controlled by a number of transcription factors that aid in lineage specification as well as lineage commitment (Pridans et al., 2008). The transcription factor Pax5 is implicated in the commitment of lymphoid progenitors to the B-cell lineage. The Pax5 gene encodes the B-cell-specific activator protein (BSAP). Pax5 is first expressed in the B-cell lineage at the pro-B cell stage, remains highly expressed in the mature B-cells, and is repressed during plasma cell differentiation. In order for a B-cell to develop normally, Pax5 must be constitutively expressed. Previous research indicates that Pax5 inactivation can cause mature B cells to convert into T cells via dedifferentiation into uncommitted progenitors. Thus, Pax5 is considered the “guardian” of both the identity and function of B-lymphocytes (Cobaleda et al., 2007).

Research suggests that the stepwise activation of Pax5 in early lymphopoiesis may provide insights into the process of B cell commitment. Many additional factors are
implicated in regulating the Pax5 gene including PU.1, IRG4, IRF8, and NF-κB, Blimp-1 and Xbp-1. Additionally, researchers have proposed a role for EBF1 in inducing chromatin accessibility in the Pax5 promoter region (Decker et al, 2009). Of the factors regulating Pax5 during terminal B-cell differentiation, the two most important ones are Blimp-1 and Xbp-1. During B-cell activation, the induction of Blimp-1 causes the reduction in Pax5 levels. Blimp-1 also causes immunoglobulin expression to shift from the membrane to secreted form, leading to increased levels of secreted immunoglobulin and decreased levels of membrane bound immunoglobulin. Xbp-1 expression is repressed in mature, resting B cells, but is de-repressed as a result of Pax5 down-regulation by Blimp-1. Xbp-1, along with Blimp-1, is necessary to drive B-cells toward the plasma cell stage. An important marker for B-cell activation is the ratio of secreted to membrane immunoglobulin because this ratio is very low in mature B cells and is increased significantly in activated B-cells and plasmablasts. Plasma cells lack membrane bound immunoglobulin altogether and have the highest ratio of secreted to membrane IgM. In rainbow trout, the transcription factors EBF, Pax5, Ikaus, Blimp1, and Xbp1, have been identified as well, and likely play similar roles in B cell development and activation (reviewed in Zwollo et al., 2011).

The structure of Pax5 is important in determining protein function.

In biology, structure is closely related to function. The Pax5 gene is 190-kb-long and is transcribed from two separate promoters (Figure 3). This gives rise to two distinct mRNAs by alternative splicing of exon 1 at the 5’ end of the gene (1A and 1B) onto the common coding sequences of exons 2–10 (Busslinger et al. 1996). The full-length vertebrate Pax5 protein is 391 amino acids in size (Adams et al., 1992). Pax proteins
bind to target genes via the paired domain, the only DNA binding domain in Pax5. The paired domain of the Pax5 gene is encoded by exons 2 and 3, and spans 128 amino acids (Chi and Epstein, 2002).

**Figure 3:** The structure of the full-length Pax5 gene. The transactivation domain is found in exon 9 and exon 10 while the paired domain is found in exon 2 and exon 3. There are two start sites in this gene, one in exon one and a second in exon 3 (Zwollo et al., 1997).

Alternative splicing allows one gene to function in diverse ways.

Alternative splicing is the splicing of the same pre-mRNA two or more ways which allows one gene to encode for two or more different mRNAs, which in turn leads to two or more protein products. Previous research has found that the Pax5 gene is alternatively spliced during B-cell development in mouse and humans (Zwollo et al., 1997). The first research in the area of Pax5 alternative splicing discovered the presence of Pax5 isoforms Pax-5a, Pax-5b, Pax-5d, and Pax-5e in mice. Pax-5a is the full-length isoform while Pax-5b and Pax-5e have their second exon spliced out. Isoforms lacking exon 2 will be referred to as Pax5Δ2 (Figure 4).

In mice, Pax5Δ2 protein is undetectable in pro-B-cells but becomes detectable during later stages of B-cell development and therefore has been stated to correlate with the differentiation stage of B-cell lines (Zwollo et al. 1997). Based on this expression
pattern, Pax5Δ2 has been hypothesized to play a regulatory role in transcription. When exon 2 is spliced out, the resulting mRNA only contains 214 nucleotides from exon 3. The transcripts lacking exon 2 use the second AUG for translation, due to a stop codon in exon 3, and as a result, only a very small part of the DNA binding domain remains in delta2 Pax5 proteins. Not surprisingly, previous research has shown that Pax5Δ2 does not bind to DNA in vitro (Zwollo et al. 1997).

In addition to having a partial DNA-binding domain, exon 2–lacking isoform Pax-5e has also deleted the 3’ region which contains the transactivation domain and replaced it with a novel sequence. This splicing changes the transcription factor’s ability to bind to target DNA and thus its ability to alter gene expression. Pax-5a is expressed in pro-, pre- and mature B-cell lines while Pax-5b is present at low levels in mature B-cell lines but not in pro-B or T-cell lines. Pax-5a and Pax-5d are able to interact with a protein binding site on the blk promoter. This gene encodes a protein tyrosine kinase that associates with the B-cell antigen receptor complex and expression is restricted to B-lymphoid cells. Pax-5b and Pax-5e could not bind to the promoter. From this study, researchers concluded that differential expression of alternatively spliced Pax-5 isoforms could be integral regulators of transcription during B-cell development (Zwollo et al., 1997).

The Pax5Δ2 isoform may be able to interact with other regulatory proteins, even though it cannot bind to the BSAP site, and could function as a co-repressor by competing with Pax5FL for binding to regulatory molecules. Alternatively, Pax5Δ2 could be a co-activator through interaction with other transcription factors (Zwollo et al., 1997).
Figure 4: Structure of the Pax5Δ2 isoform. Note that Exon 2, missing in this isoform, is a component of the Paired Box domain of the Pax5 gene (Zwollo). The resulting protein is shorter as it starts at AUG2 (see figure 3).

Studies conducted at the Atlantic Cancer Research Institute state that in normal, human B cells, there are multiple isoforms of Pax5, including isoforms lacking exon 2, as well as in B-cell lymphoma and chronic lymphoid lymphoma cells. These isoforms were not deregulated, but instead showed complex patterns of expression (Arseneau et al., 2009). It has been observed that multiple myeloma patients have higher Blimp1 levels and lower levels of Pax5 compared to healthy donors, which suggests that the mature B cells in these patients are in a more activated state (Borston et al., 2002). Increased levels of alternative splice forms are also seen in these patients. Thus, abnormal expression of alternative Pax5 splice forms may cause resting B-cells to go into an activated state by preventing repression of Blimp1 (Borston, et al., 2002).

Our lab discovered recently that the rainbow trout Pax5 gene is also extensively alternatively spliced, including isoforms lacking exon 2 (unpublished results). This honors thesis research aimed to investigate the expression of Pax5Δ2 in B-cells of LPS activated rainbow trout B cells, and in B cells of spawning Oncorhynchus nerka (O. nerka).

Spawning and Immunity in Salmon

Oncorhynchus nerka are anadromous salmonoid fishes that spend part of their life in the ocean. After having spent 1 – 3 years feeding and growing in the Pacific Ocean, O. nerka will return to the site from which they were conceived. Therefore, they must
power their way back to their birthplace to spawn, even though they ate their last meal at
sea and will starve throughout their journey (Hill, p.3). For a spawning Pacific salmon,
there is a huge cost to reproduction. The fish will die once it reaches its spawning
grounds and releases its eggs. The fish, however, must stay alive during this journey.
More importantly, the fish must not only stay alive, but must remain reproductively
viable. The question then is what happens with the immune system that enables these
fish, under such intensely harsh conditions to survive and reproduce. An understanding
of this process requires knowledge about the stress response in these animals and how
that stress affects both the endocrine and immune systems. At this point, it is important
to also note that different taxa of fish have different stress tolerances, making this a very
difficult field of study (Schreck et al, 2001).

Schreck et al. define stress as the “physiological cascade of events that occurs
when the organism is attempting to resist death or reestablish homeostatic norms in the
face of an insult” (Schreck et al, 2001). While stress is generally believed to cause
reduced immune capacity, a recent study in mice demonstrated that when these animals
were subjected to multiple bite wounds they became resistant to the immunosuppressive
effects of stress and glucocorticoids (Avitsur et al., 2000). Avitsur et al. propose that this
response is adaptive because it is optimal to maintain immune defenses in the face of
numerous wounds. Because fish encounter a number of obstacles (numerous wounds) on
their way to their spawning grounds, an adaptive response similar to the one described by
Avitsur et al. seems likely during spawning in fish. It is apparent that stress and the
immune system are closely related. A more thorough understanding of this relationship
requires an understanding of how key hormones modulate immunity in fish.
For the purpose of this study, it was important to have a full understanding of the effects of cortisol on B-cells. Cortisol is the primary corticosteroid produced during a stress response in the teleost interrenal gland; the interrenal gland of fishes is intermingled with other tissues. More specifically, both steroidogenic (corticosteroid-producing) and chromaffin (chatecholamine-producing) cells are scattered along the posterior cardinal vein and the hematopeoitic head kidney (anterior kidney) (Evans and Claiborne p. 288, Harris and Bird, 2000). Administration of cortisol to teleost fish has been shown to reduce the number of circulating B and T lymphocytes. It has been hypothesized that during a stress response, these cells are trafficked to lymphoid tissues such as the anterior kidney. Additionally, cortisol has been shown to induce the apoptosis of B cells in carp lymphocytes (Weyts et al., 1997). Wojtaszek et al. observed lymphopenia in the blood: after 24 hours there was a reduction from 63% to 20% in percent lymphocytes when cortisol was injected into the blood of carp (Wojtaszek et al., 2002). Maule and Schreck, studying O. kisutch, also noticed a decrease in the frequency of leukocytes in spleen and PBL, as well as increase in the anterior kidney, in response to acute stress or cortisol in food (Maule and Schreck, 1990). Both lymphopenia and apoptosis of B cells could account for their clearance from the blood following stress. Cortisol has also been shown to reduce levels of phagocytosis and lymphocyte proliferation, as well as the activity of immunoglobulin producing cells and circulating levels of IgM (Harris and Bird, 2000).

In goldfish, there do not appear to be any immunosuppressive symptoms during spawning (Suzuki et al., 1996). Yet, during spawning season, rainbow trout have decreased numbers of antibody secreting cells and lower IgM serum titers (Hou et al.,
A study by Weyts et al. determined that increased cortisol correlates with increases in apoptosis of LPS-activated, but not resting, B lymphocytes in PBL (Weyts et al., 1998).

In carp, the number of IgM secreting cells in PBL, spleen, and anterior kidney are surprisingly high when cortisol levels are high (Saha, 2002). These data are surprising because cortisol can induce apoptosis in the absence of a stimulant in carp (Saha, 2003). After incubation with 100ng/ml cortisol for 12 hours, ELISPOTS show the number of IgM secreting cells decreases in all three tissues (Saha, 2004). ELISA analysis of IgM titer shows two days after cortisol incubation, there is a 15 – 20% decrease in all three tissues. The reduction in antibody secreting cells may reflect an inhibiting effect of cortisol on proliferating B-cells (plasmablasts), but it is possible that the plasma cells are not affected by cortisol (Saha, 2004). Glucocorticosteroids are thought to induce apoptosis by crosslinking IgM (Saha, 2003), which would explain why plasma cells, which do no longer express IgM on the cell surface, are less sensitive to cortisol.

A human study exploring the effects of cortisol on B-cells shows that cortisol has an inhibiting effect on B lymphopoiesis and inhibits the formation of pro- and pre-B cells. Immature B-cells, in the presence of cortisol are apoptotic, and resting B-cells are less affected by cortisol. The study, however, did not describe the effects of very high levels of cortisol on resting B-cells (Igrashi et al., 2005).

Rahman et al. studied the relationship between prednisolon, a glucocorticoid, and Pax5 in human B-cell lines. While glucocorticoids are shown to induce apoptosis and arrest growth of pre-B cell lines (in agreement with the data presented above), prednisone
was shown to have no apoptotic effect on mature B, or pro-B cell lines. Additionally, Pax5 protein and transcript levels are decreased after growth of pre-B cell lines in glucocorticoid medium, but no glucocorticoid response element was found on the Pax5 promoter (Rahman et al., 2001). This may point to cortisol effecting B-cells through the glucocorticoid receptor that is present on B-cells.

Based on the preceding studies, it is apparent that cortisol, and other glucocorticoids, have a profound impact on B-cells. All developing B-cells are lost through inhibition of B lymphopoiesis, while pre-B cells, activated B-cells and possibly plasmablasts are lost through apoptosis. Plasma cells and mature B-cells do not appear to be as profoundly affected by cortisol and may remain, even after an organism experiences acute stress. Additionally, based on the study by Rahman et al. it is clear that Pax5 is affected by glucocorticoids.

Growth hormone and prolactin are two additional hormones produced during a stress response that have been shown to modulate the immune system in fish. In fish, growth hormone is responsible for osmoregulation as well as steroidogenesis while prolactin is functionally important for growth, osmoregulation, and reproduction. Growth hormone has been shown to stimulate lymphopoiesis, phagocytosis, and immunoglobulin production in fish. Additionally, prolactin has been shown to increase IgM levels in fish (Harris and Bird, 2000).

Reproductive hormones are also known to influence the immune system in fish (Harris and Bird, 2000). While androgens are primarily associated with male humans, in fish, testosterone and androstenedione are major products of the female ovaries. In fish,
testosterone has immunosuppressive effects. It reduces the number of immunoglobulin producing cells and has been shown to kill leukocytes in vitro (Slater and Schreck, 1997). Direct counts of viable leukocytes after incubation with and without testosterone demonstrate that testosterone leads to a significant loss of leukocytes. To induce significant immunosuppression, at least five days of contact with testosterone were required. Addition of media from proliferating lymphocytes not exposed to testosterone was not able to reverse the immunosuppressive effects of the testosterone. Thus, testosterone may exert its immunosuppressive effects through direct action on salmonid leukocytes (Slater and Schreck, 1997).

During the spawning process, salmonoids display high levels of reproductive hormones, including testosterone. Both male and female salmonoids display immune deficiencies during this stage of their life cycle. A study of Pacific salmon during spawning found positive correlations between plasma reproductive steroid levels and lysozyme activity and the number of immunoglobulin producing cells (Harris and Bird, 2000). This suggests that both the innate and adaptive immune system (humoral immunity) is activated during spawning.

From this brief overview of just three different hormones, it is clear that stress has a dramatic effect on the immune system of fish. The study of the effects of hormones on the fish immune system is very complicated and will take many years to fully elucidate. However, it is known that hormones produced during reproduction and stress affect the immune system and have important implications for fish health.
Quantitative Real-Time PCR

Polymerase chain reaction (PCR) was developed in 1983 by Kary Mullis and is now a widely used technique that amplifies specific sequences of DNA. Advancements in PCR technology combined with fluorescent technology led to the development of quantitative or real-time PCR. Quantification of cDNA in real-time PCR is based on the measurement of the increasing fluorescence intensity after each PCR cycle (Ramackers, 2002). Measuring the kinetics of the reaction starting at the early phases of PCR provides a distinct advantage over traditional PCR detection which uses agarose gels for the detection of the PCR product at the end-point of the reaction. The problems with end-point detection include: not quantitative, poor precision, low sensitivity, low resolution, size based discrimination only, and results that are not expressed as numbers. To further understand the limitations of end-point PCR, it is important to understand the key phases of a PCR reaction. The reaction has three phases. There is the exponential phase where, assuming 100% efficiency, there is exact doubling of the product. There is the linear phase where the reaction components are consumed and the reaction is slowing. Lastly, there is the plateau, where the reaction has stopped and if left long enough the product will degrade. The plateau is what is measured with traditional end-point PCR methods (Figure 5). Measurements taken at the plateau phase do not truly represent the starting amounts of target material (ABI).
Figure 5: Logarithmic view of PCR phases (ABI).

Real-time PCR measures the amount of amplicon during the exponential phase of the PCR reaction making the quantitation of DNA both easier and more precise than with traditional methods. This Honors research used SYBR Green Dye to perform real-time analysis. SYBR Green dye binds to the minor groove of double stranded DNA (Figure 6). As more amplicons are produced, the intensity of the fluorescence increases. The main drawback to SYBR Green Dye is that it is not as precise as other available probes because instead of binding to a specific sequence of DNA, SYBR green will bind to any DNA that is double stranded. This can cause issues if the primer sticks to itself forming a primer dimer (ABI).

Figure 6: SYBR Green binding (NFSTC).
Thus, while setting up a real-time assay can be a challenge, there are a number of advantages to using Real-Time PCR over traditional PCR. First, Real-Time PCR collects data in the exponential growth phase while traditional PCR measures a plateau. Second, an increase in fluorescent signal is directly proportional to the number of amplicons produced by the reaction. Third, cleavage of the probe provides a permanent record of amplification of the amplicon; thus, degradation of the final product does not lead to inaccurate data. Finally, Real-Time PCR is capable of detection down to a 2-fold change making it much more precise than the traditional end-point method with agarose gel (ABI).

**Research Question, Hypothesis, and Relevance**

**Research Questions**

1. Are there changes in the alternative splicing of Pax5 during a humoral immune response in the genus *Oncorhynchus*?

2. Specifically, what changes are seen in *Oncorhynchus* Pax5 isoforms that lack exon 2 and does this correlate with an increase in the levels of secreted IgM?

**Hypothesis**

There are changes in alternative splicing of Pax5 during a humoral immune response in the genus *Oncorhynchus*. Specifically, as the levels of Pax5Δ2 increase, the levels of secreted IgM also increase. Pax5Δ2 is acting as co-repressor of Pax5FL.

This project aimed to use quantitative real-time PCR to determine whether there is a relationship between the alternative splicing of Pax5 and a humoral immune response in *Oncorhynchus*. The project used both an *in-vivo* and an *in-vitro* model to explore this
relationship. For the in-vitro model, we stimulated rainbow trout B cells with LPS. As the cells become stimulated, we predicted that the level of secreted IgM would increase and, based on this hypothesis, the levels of Pax5Δ2 would also increase. This prediction was based on the work by Zwollo et al. stating that Pax5Δ2 may act as a co-repressor of Pax5FL (Zwollo et al. 1997). Additionally, for the in-vivo model, immune tissues from O. nerka were collected from different locations on the path of fish to the spawning grounds. We hypothesized that the fish would maintain some level of humoral immune response as they get closer to their spawning ground in order to maintain their health so as to not lose their reproductive abilities. An increase in the levels of secreted IgM was predicted to cause an increase in the level of Pax5Δ2 expression.

Our hypothesis that Pax5Δ2 is acting as a co-repressor of Pax5FL is, in part, based on a study by Lowen et al. that illustrates that Pax5FL modulated by alternatively spliced isoforms. Through comparison of various B-cell lines and resting and LPS activated mature B-cells, Lowen et al. concluded that increased B-cell proliferation correlates with increased levels of Pax-5e, an isoform missing both exon 2 and exons 6-10 (transactivation domain). Conversely, they also demonstrated that increased Pax-5d, an isoform missing exons 6-10, correlated with inhibition of cell growth. These findings together led the researchers to conclude that during activation and differentiation of B-lymphocytes, Pax5FL is modulated by alternatively spliced isoforms (Lowen et al., 2001). The current honors research aimed to determine how Pax5Δ2 fits into this picture.

Determining the expression patterns and ultimately the function of the Pax5Δ2 isoform is interesting because this isoform is missing a portion of the paired domain. Because of the missing exon in the paired domain region, the isoform may have altered
co-transactivating or co-repressive capabilities, which implies that the isoform has interesting and important functions in B-cells. Studies performed on the Pax8 and Pax6 genes indicate that isoforms with altered paired domains lose sequence specificity and are subsequently able to bind to other areas with greater affinity than their traditional binding site. However, the presence of a homeodomain (Pax 6) can help to stabilize the weak DNA interactions of the Paired Domain while proteins lacking a homeodomain (Pax 8) may be able to interact with other proteins if the Paired Domain has been altered (Kozmik et al, 1997).

This research could have a number of clinical applications specifically in the area of lymphomas. The human Pax5 gene has been implicated as a tumor suppressor or as an oncogene in leukemias as well as non-Hodgkin lymphomas and other lymphoid malignancies (Decker et al, 2008). Discovery of a Pax5 enhancer in the intron of exon 5 provides insight into the role of Pax5 cis-regulatory elements in chromosomal translocations that are implicated in the development of B-cell precursor acute lymphoblastic leukemia (BCP-ALL). In BCP-ALL, translocations of Pax5 generate novel transcription factors through the fusing of the N-terminal DNA-binding domain of Pax5 to the C-terminal sequences of other transcription factors, kinases, and structural proteins (Decker et al, 2009). Additionally, research conducted in CLL patients established that the predominant expression of full length Pax5 is generally accompanied by minor expression of various isoforms and that most of these isoforms are indeed translated into proteins. Therefore, we have a clear link between Pax5 isoforms and lymphomas indicating that this research could prove to be very important from the clinical perspective.
Recent advances in mammalian Pax5 studies suggest that Pax5 is able to activate the transcription of human telomerase reverse transcriptase gene in B cells. This is important because it suggests that in addition to regulating the development and activation of B-cells, Pax5 can affect the immortality of a cell (Bougel et al, 2009). It would be very interesting to determine if the various isoforms of Pax5 play any role in regulating cell immortality. This would be important news in the cancer research world.

Lastly, this study furthers our understanding of how salmonoid species are able to travel thousands of miles from salty water to fresh, enduring attack by thousands of parasites and diseases, and still have viable gametes with which to reproduce at their final destination. A deeper understanding of this process could then be applied in a comparative physiology context.
Methods

Animals and facilities

Outbred rainbow trout (200-500 grams) were gifts from Dr. Steve Kaattari (Virginia Institute of Marine Science). Fish were maintained in a 100-gallon tank with a re-circulating system employing filtered well water. Fresh water exchange was approximately 10% per day. Water temperature was maintained at 12°C. Fish were fed dry, floating pellets (Aqua Max Grower 600, Purina) once a day.

Tissue Collection and Storage

Immune tissues from wild sockeye salmon (*Oncorhyncus nerka*) were collected by Dr. Zwollo from four sites in South Central Alaska, in accordance with an approved IACUC protocol. These included the Main Bay (Prince William Sound), Mouth of the Kenai River, Russian River, and Quartz Creek-Upper Kenai River (Figure 7). Live fish were donated by local fishermen, and dissected immediately by Dr. Zwoll. Approximately 0.3-0.5 cubic cm samples from spleen, anterior kidney and posterior kidney were collected into 750-1000ul ul of RNAlater reagent (Applied Biosystems Inc.) and stored overnight at 4C, then frozen at -20C until use. 100 µl blood samples were collected into 750 µl RNA later (Ambion).
Rainbow trout were anesthetized with ethyl-3-aminobenzoate methanesulfate salt (Sigma) in accordance with our IACUC protocol. Blood was collected in heparinized tubes and spleen tissues in 5 mls sterile HBSS (137 mM NaCl, 5.6 mM D-glucose, 5 mM KCl, 8.1 mM Na$_2$HP0$_4$.$2$H$_2$O, and 20 mM Hepes at pH 7.05). Single cell spleen suspensions were obtained by repeated uptake and release through a 10 ml syringe followed by forcing cells through a 40 nm nylon cell strainer (Falcon/BD Biosciences). Erythrocytes were removed using Histopaque 1077 cushions (Sigma Aldrich) and spun at 500g at 4 °C for 30–45 min and cells collected in HBSS or PBS (1.9 mM NaH$_2$P0$_4$.$H_2$O, 8.1 mM Na$_2$HP0$_4$.$7$H$_2$O, 137 mM NaCl, and 2.6 mM KCl, pH 7.4) containing 0.02% sodium azide (PBS-SA).
**LPS Activation**

Freshly isolated splenic trout cells with erythrocytes removed were cultured in trout culture medium (TCM) consisting of RPMI 1640 with 10 mM L-glutamine, 10% Fetal Calf Serum, 50 µg/ml gentamicin, 50 µM 2-ME, and the nucleosides adenosine, uracil, cytosine, and guanosine (10 µg/ml; Sigma-Aldrich) at 18°C and in the presence of blood gas (10% CO₂, 10% O₂, 80% N₂). Cells were activated using lipopolysaccharide (LPS) (055:B5 from E.coli; Sigma) at 100 µg/ml. Cells were fed every other day with one tenth of the culture volume of a 10x tissue culture cocktail containing 500 µg/ml gentamycin, 10x essential amino acids (aas), 10x non-essential aas, 70 mM L-glutamine, 70 mg/ml dextrose, 10x nucleosides, and 33% FBS. Cells were collected on the indicated days and frozen at -80°C until RNA purification.

**RNA Extraction**

RNA was extracted using the TRIzol Reagent (Invitrogen). 50 mg of tissue was homogenized into 2ml of TRIzol using a Fisher PowerGen 125. Next, due to the high content of proteins in the samples, following homogenization samples were spun at 12000g for 10 min to remove insoluble material. The cleared homogenate solution was transferred to a fresh tube and 0.4 ml of chloroform was added to remove soluble protein. Samples were vigorously shaken for 15 seconds. Following a 3 min incubation at room temperature and a 10 min spin at 12000g, the solution was separated into a red phenol-chlororophorm phase, an interphase containing protein, and a colorless upper aqueous phase containing the RNA. The aqueous phase was transferred to a fresh tube and one-half a volume (1ml) isopropyl alcohol added to precipitate the RNA. Samples were incubated at room temperature for 10 min and then spun at 12000g for 10 min. The RNA
precipitates as a gel-like pellet on the side and bottom of the tube. Subsequently, the RNA was washed using 2 mls 75% ethanol, to remove excess salts, the tube vortexed, and spun at 7500g for 5 min. The RNA pellet was then air-dried for 10 min and resuspended in 40µl RNase-free water. RNA concentration was determined using the NanoDrop™ ND-1000 full-spectrum (220nm-750nm) spectrophotometer. RNA quality was checked by gel electrophoresis.

**cDNA and Real-Time PCR**

1µg of total RNA was used in a 20µL volume to make cDNA using the iScript kit (BioRad). Quantitative real-time PCR was performed using a PerfeCta SYBR Green SuperMix, ROX PCR kit (Quanta Biosciences) with a reaction mix containing 12.5µL SYBR Green, 9.5µL RNase free water, 1µl each primer (Table 1 and Figure 8), and 1µL cDNA in 25µL of total reaction volume on an Applied Biosystems StepOne Real-Time PCR instrument. As a means of quality control for each run, negative controls were included in each plate set-up where cDNA template was substituted by equal amount of nuclease free water. Each RNA template, before being transcribed to cDNA was used in equal concentration in PCR reaction as a means to ensure no PCR amplification of possible leftover genomic DNA in the sample. Initial run analysis was performed using StepOne Software Version 2.1.

Before beginning the real-time PCR experiments, a validation experiment was performed to verify that the efficiency of the reference (tubulin) primer and target primers were approximately equal. To assess the efficiency of the primers, serial dilutions of target and tubulin primers were prepared and real-time PCR was performed.
The $C_T$ values for each dilution for target and tubulin were obtained and the $\Delta C_T$ value was calculated. Next, a plot of the log input amount versus the $\Delta C_T$ value was prepared. A slope of approximately zero indicates that the efficiencies of the two amplicons are approximately equal (Dorak).

To confirm that only one product of the correct size was being amplified by the primers, the products from a real-time PCR reaction were run on a 2% agarose gel. The first well contained a 1kb ladder and the remaining wells contained 5µL of 6X dye and 25µL of PCR product. The gel was run at 70mV for 1 hour.

Additionally, to confirm that the *Onchorynchus* spp. Pax5 was being amplified by the primers, the real-time PCR fragment was sequenced. Direct sequencing was performed by capillary electrophoresis on ABI 3130 Avant Genetic Analyzer. Sequencing PCR reactions were carried out with BigDye Version 3.1 chemistry in both direction. BioEdge columns were used in PCR clean-up before denaturing purified samples in HiDi formamide. Samples were resolved on a 36cm capillary and Pop7 polymer. Sequences were analyzed on ABI sequencing software version 5.1. An NCBI Blast tool was used to align obtained sequences with the published *Onchorynchus* spp. sequence data.

PCR was run under the following conditions for the secreted (qt.HCmu.sec.AS and qtHCmu.S) and membrane IgM (qtHCmu.mem.AS and qtHCmu.S): cDNA was denatured at 95.0 °C for 10 min. After this, sample was exposed to 95.0 °C for 15sec and 60 °C for 1 min for 40 cycles in a two-step PCR reaction. Finally, a one-time increase to 95 °C for 15 sec generated the melt curve. The reaction was run under the following
conditions for the Pax5FL and Pax5Δ2 primers: cDNA was denatured at 95.0 °C for 10 min. After this, sample was exposed to 95.0 °C for 15 sec and 56 °C for 1 min for 40 cycles in a two-step PCR reaction. Finally, a one-time increase to 95 °C for 15 sec generated the melt curve which served as a quality control measurement. Each sample was measured in triplicate. Tubulin, PaxFL, and PaxΔ2 were run together in one assay while tubulin, secreted, and membrane IgM were run together in another assay. With the 48-well machine, five samples were run with three primer sets in each reaction.

**Table 1:** Primer Information

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Target</th>
<th>Primer Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>qtHCmu.S</td>
<td>Secreted and Membrane IgM</td>
<td>5’-GCG CTG TAG ATC ACA TGG AA-3’</td>
</tr>
<tr>
<td>qt.HCmu.sec.AS</td>
<td>Secreted IgM</td>
<td>5’-GCA AGT CAG GGT CAC CGT AT-3’</td>
</tr>
<tr>
<td>qtHCmu.mem.AS</td>
<td>Membrane IgM</td>
<td>5’-TTT CAC CTT GAT GGC AGT TG-3</td>
</tr>
<tr>
<td>Ttub60.F</td>
<td>Tubulin</td>
<td>5’-CGT CCC CAG GTG TCC ACT-3’</td>
</tr>
<tr>
<td>Ttub60.R</td>
<td>Tubulin</td>
<td>5’-GTA GGT GGG GCG CTC AAT-3’</td>
</tr>
<tr>
<td>tPax5/E3-4.AS</td>
<td>Pax5FL and Pax5Δ2</td>
<td>5’-CCT GAT GAT CCT GTT GAT AGA-3’</td>
</tr>
<tr>
<td>tPax5E2.S</td>
<td>Pax5FL</td>
<td>5’-CAC GGA TGT GTC AGC AAG AT-3’</td>
</tr>
<tr>
<td>tPax5/E1-3.S</td>
<td>Pax5Δ2</td>
<td>5’-CGG GAC GAG CAG GTA CTA TG-3’</td>
</tr>
<tr>
<td>Ttub56.F</td>
<td>Tubulin</td>
<td>5’-TTC ACC TCC CTG CTG ATG-3’</td>
</tr>
<tr>
<td>Ttub56.R</td>
<td>Tubulin</td>
<td>5’-CCA CCA TGA AGG CAC AGT-3’</td>
</tr>
</tbody>
</table>
Figure 8: Primer Location. This figure illustrates where each primer binds. The Pax5FL product produced by primers tPax5E2.S and tPax5/E3-4.AS is 237 base pairs in length while the Pax5de2 product produced by primers tPax5/E1-3.S and tPax5/E3-4.AS is 198 base pairs in length.

Analysis of Quantitative Real-Time PCR

After the reaction was complete, C_T values provided by the real-time PCR instrument were exported as a .txt file onto a USB drive. The data were then uploaded into DataAssist Software (ABI). The Pax and Sec/Mem assays had to be analyzed separately. Multiple experiments were combined in one DataAssist study to enable analysis of all 2009, 2010, and OM samples to be studied together, respectively. Expression levels were calculated as fold-change relative to the Day0 fish for LPS activated cells and to the reference sample (averaged O. mykiss C_T) for the O. nerka
samples. Expression of individual genes from each sample was normalized to relative expression of trout $\alpha$-tubulin within the same experiment. Results from DataAssist Software were exported to Excel for graphing and statistical analysis.

In DataAssist, for each study, PaxFL, PaxΔ2, Secreted, and Membrane were selected as ‘Targets’ and Tubulin as a ‘Selected Control’ in the ‘Assay Design’ section of the form. In the ‘Analysis Settings’ box, the maximum allowable $C_T$ value was set at 35.0, ‘Yes’ was selected in the ‘Include max$C_T$ values in calculations’, the box for ‘Exclude outliers among replicates’ was checked, ‘Arithmetic Mean’ was selected as the normalization method, and the Reference Sample was selected based on the assay being analyzed (averaged values of the OM Samples for all *O. nerka* data). After pressing “Perform Analysis” the software generates the average $C_T$, $\Delta C_T$, and fold change values for each sample. Results from DataAssist Software were exported to Excel for graphing and statistical analysis.

The following equation was used by DataAssist to calculate the $\Delta C_T$ (1).

$$\Delta C_T = C_{T,\text{Target}} - C_{T,\text{Tubulin}}$$

The $\Delta \Delta C_T$ value was calculated separately in an Excel spreadsheet (2):

$$\Delta \Delta C_T = (C_{T,\text{Target}} - C_{T,\text{Tubulin}})_{\text{SampleX}} - (C_{T,\text{Target}} - C_{T,\text{Tubulin}})_{\text{Ref Sample}}$$

Finally, the fold change, or amount of target, was calculated by DataAssist according to the following equation (3) (Livak and Schmittgen, 2001).

$$\text{Fold Change} = 2^{-\Delta \Delta C_T}$$
The ratio of Fold Change Secreted/Fold Change Membrane as well as the ratio of Fold Change Pax5FL/Fold Change PaxΔ2 was calculated in an Excel spreadsheet. These ratios were then input into a scatterplot to calculate the slope of the line and the correlation coefficient in order to determine if there was a correlation between the alternative splicing of Pax5 and an immune response in these samples (See Attachment 1 for more information on the ΔΔCT method).
Results

The research presented in this thesis had the following two goals. The first goal was to determine if there are changes in the alternative splicing of Pax5 during a humoral immune response in teleost fish. Specifically, the aim was to determine what changes are seen in the levels of both the full length Pax5 form and any isoforms that lack exon 2 (delta 2) and whether these changes correlate with an increase in the levels of secreted IgM. The second goal aimed to determine if there are changes in Pax5 delta2 usage during the spawning period of Oncorhyncys nerka, and whether this correlates with the increased antibody response observed during spawning. To do this, we initially measured IgM and Pax5 transcript levels in Oncorhynchus mykiss immune tissues during an in-vitro LPS response, to provide a controlled system to study how Pax5 alternative splicing is altered during an immune response. Two real-time PCR assays were designed and used to analyze samples. Next, this technique was then used to analyze immune samples collected from four locations along the O. nerka spawning grounds in South Central Alaska. The following section describes the data collected over the course of the study.

Observation of Fish Location and Health

To obtain a full understanding of the relationship between alternative splicing and an immune response as a result of spawning, it was important to take detailed notes on both on the location and health of each fish. These notes also served as a point of comparison with the qPCR data. As the fish were collected, they were assigned a number, which was used in the remainder of the project. The location where the fish was
caught was also noted. This location became very important as we assessed whether alternative splicing changes during the spawning process. Finally, a brief description of each fish was documented. This description included sex and overall health and any other characteristics seen in specific organs. See Tables 2A and 2B provide a full description of each fish.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Location Caught</th>
<th>Description of Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>51</td>
<td>Mouth Kenai River</td>
<td>Female, large spleen, lots of worms around eggs</td>
</tr>
<tr>
<td>52</td>
<td>Mouth Kenai River</td>
<td>Female, regular size spleen, healthy, worms around eggs, bloody PK</td>
</tr>
<tr>
<td>53</td>
<td>Mouth Kenai River</td>
<td>Male, regular size spleen, looked healthy, large fish, grainy brown PK (aged?), no worms</td>
</tr>
<tr>
<td>54</td>
<td>Russian/Kenai River</td>
<td>Male, healthy, some worms, bloody PK, small spleen</td>
</tr>
<tr>
<td>55</td>
<td>Russian/Kenai River</td>
<td>Male, healthy, no worms, good PK and AK, spleen average size</td>
</tr>
<tr>
<td>56</td>
<td>Russian/Kenai River</td>
<td>Female, small spleen, large fish, grainy brown PK, AK, and SPL</td>
</tr>
</tbody>
</table>

Table 2A – List of samples from 2009 fish collection.

<table>
<thead>
<tr>
<th>Sample Name</th>
<th>Location Caught</th>
<th>Description of Fish</th>
</tr>
</thead>
<tbody>
<tr>
<td>101</td>
<td>Main Bay</td>
<td>Female, healthy, no spawning colors</td>
</tr>
<tr>
<td>102</td>
<td>Main Bay</td>
<td>Female, bite marks, infection on abdomen, appeared healthy overall, no spawning colors</td>
</tr>
<tr>
<td>103</td>
<td>Main Bay</td>
<td>Female, average size, healthy, no spawning colors</td>
</tr>
<tr>
<td>104</td>
<td>Main Bay</td>
<td>Female, average size, healthy, no spawning colors</td>
</tr>
<tr>
<td>105</td>
<td>Mouth of Kenai River</td>
<td>Male, large, healthy</td>
</tr>
<tr>
<td>106</td>
<td>Mouth of Kenai River</td>
<td>Male, small, worms, mottled liver, grainy kidney, regular size spleen</td>
</tr>
<tr>
<td>107</td>
<td>Mouth of Kenai River</td>
<td>Male, healthy, no worms, big spleen</td>
</tr>
<tr>
<td>108</td>
<td>Russian River</td>
<td>Female, many worms, small eggs, whole body net marks, small spleen</td>
</tr>
<tr>
<td>109</td>
<td>Russian River</td>
<td>Male, large, small spleen, no worms</td>
</tr>
<tr>
<td>110</td>
<td>Quartz Creek-Upper Kenai River</td>
<td>Spawned Female, lots of worms, no eggs, stomach cavity jellied, hard small spleen with granules</td>
</tr>
<tr>
<td>111</td>
<td>Quartz Creek-Upper Kenai River</td>
<td>Male - spawned but still some milt loose in stomach cavity, only a few worms</td>
</tr>
<tr>
<td>112</td>
<td>Quartz Creek-Upper</td>
<td>Male - bite mark in right shoulder area, few worms,</td>
</tr>
</tbody>
</table>
RNA Quality Control

For real-time PCR applications, RNA quality is vitally important for obtaining accurate results. The assay’s sensitivity may be reduced and the relative differences in transcripts may produce incorrect target ratios if RNA is partially degraded or has impurities (Bustin et al., 2009). The RNA concentration and purity of all samples was tested by spectroscopy using the NanoDrop prior to cDNA synthesis and use in qPCR. Due to the tissue collection and the inherent nature of immune tissue, high quality RNA was difficult to achieve. After testing various RNA extraction protocols, including Quiagen Kits and Trizol reagent, Trizol reagent was found to be most effective for obtaining high quality RNA for quantitative PCR. DNAfree, a reagent containing DNase, was tested to try to improve RNA quality. While the reagent was able to remove DNA contamination, it also significantly reduced the RNA yields. If samples had very low 260/280 or 260/230 ratios, (the 260/280 should be higher than 1.9 and the 260/230 ratio higher than 2.0 (Jain et al. 2006)) the samples were run on a 2% agarose gel to determine if RNA was of high enough quality for use in assay. High quality RNA produces only two bands when run on a gel the bands are 18S and 28S ribosomal RNA. Low quality RNA produces a smear down the lane. Table 3 provides an example of RNA concentration and purity in this experiment (See Attachment 1 for more details about RNA in this study).
<table>
<thead>
<tr>
<th>Sample Name</th>
<th>[ng/µL]</th>
<th>A_{260}:A_{280}</th>
<th>A_{260}:A_{230}</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPL 101</td>
<td>207.87</td>
<td>1.89</td>
<td>2.11</td>
</tr>
<tr>
<td>SPL 102</td>
<td>124.60</td>
<td>1.81</td>
<td>2.32</td>
</tr>
<tr>
<td>SPL 103</td>
<td>97.59</td>
<td>1.89</td>
<td>1.92</td>
</tr>
<tr>
<td>SPL 104*</td>
<td>24.08</td>
<td>1.89</td>
<td>1.36</td>
</tr>
<tr>
<td>SPL 105</td>
<td>112.55</td>
<td>1.92</td>
<td>1.72</td>
</tr>
<tr>
<td>SPL 106</td>
<td>174.70</td>
<td>1.90</td>
<td>2.00</td>
</tr>
<tr>
<td>SPL 107</td>
<td>1911.25</td>
<td>1.99</td>
<td>1.64</td>
</tr>
<tr>
<td>SPL 108</td>
<td>2977.05</td>
<td>1.96</td>
<td>1.73</td>
</tr>
<tr>
<td>SPL 109</td>
<td>1927.51</td>
<td>1.97</td>
<td>1.74</td>
</tr>
<tr>
<td>SPL 110</td>
<td>1638.18</td>
<td>2.02</td>
<td>1.98</td>
</tr>
<tr>
<td>SPL 111</td>
<td>2262.60</td>
<td>1.96</td>
<td>1.45</td>
</tr>
<tr>
<td>SPL 112</td>
<td>1690.77</td>
<td>1.96</td>
<td>1.54</td>
</tr>
</tbody>
</table>

Table 3: RNA Quality Control NanoDrop Data. * Samples with very low concentration used 0.5µg RNA to make cDNA and then 2µL cDNA in each reaction to make amount of target the same for each reaction.

Real-Time PCR Assay Validation

A large part of completing a qPCR experiment involves designing the assay. Primers must be carefully designed to prevent non-specific binding as well as primer dimers. Additionally, the primers must be tested at a number of different concentrations and melting temperatures. The products of the reaction must be relatively small (under 200 base pairs) because of the two-step nature of the reaction, and lack of a 72 °C extension step which would be required to produce a longer product. Quality of the
reaction can be assessed by a number of plots produced by the machine. The amplification plot (Figure 9) shows the amount of background fluorescence being produced as well as the logarithmic curve produced as product is formed. The melt curve (Figure 10) is the main indicator of primer efficiency. If the curve has multiple peaks it indicates that there is unspecific binding occurring. If the curve has one peak around 70 degrees and another in the 80 degree range, this shows that a primer dimer is forming. Additionally, if the melt curve has more than one peak, it indicates that multiple products are being formed. It is important to note the temperature difference between the primer dimer and the desired product. The primers that this project used for Pax5FL amplification form a primer dimer. This means that the negative control is not negative, but because the primer dimer melts at approximately 70 degrees and the melt curve for samples was melting around 85 °C, this is not a critical problem. The multicomponent plot (Figure 11) shows the level of fluorescence as compared to the baseline and verifies that the machine is functioning properly.
Figure 9: Sample Amplification Plot. The short, jagged lines indicate background fluorescence in the reaction while the logarithmic lines show the amplification of product during the reaction.
Figure 10: Sample Melt Curve. The three different curves are produced by the three different primer sets in the reaction (Tubulin, Secreted IgM, and Membrane IgM). The single peak indicates that only one product is being formed during the reaction. The yellow-green curve is the melt curve for the Tubulin primers, the purple and pink curve is the melt curve for the membrane primers, and the teal curve is the melt curve for the secreted primers.
Figure 11: Sample Multicomponent Plot. The SYBR lines below the ROX baseline are produced by the negative control samples.

As an additional step to verify that the primers are all amplifying at approximately the same efficiency, a validation experiment was run at each melting temperature. In these experiments, all primer sets were run at $56\, ^\circ C$ and $60\, ^\circ C$ with varying concentrations of primer. The experiment verified that $1\, \mu L$ of primer in each reaction was optimal because, even though most of the primers worked very efficiently at a much
lower concentration, the Pax5FL primer set worked most efficiently with 1 µL of primer and primer concentration needs to be standardized in the assay.

**Figure 12A:** Validation assay for $T_m = 56 \degree C$. Primers were all working at approximately the same efficiency and that the assay was amplifying product optimally at $56 \degree C$ with 1 µL primer for the Pax primers and at $60 \degree C$ with 1 µL primer for the IgM primers.
To verify that the primers were amplifying the desired Pax5 products, qPCR product was sequenced and the resulting sequence was compared to published sequences in the NCBI website using the BLAST tool. Sequences were as expected based on published data (refer to NCBI Reference Sequence: NM_001124682.1).

Additionally, the products from both an IgM and a Pax real-time PCR run were analyzed using gel electrophoresis. Figure 13 verifies that each primer set is producing only one product of approximately the expected size. The Tubulin 56 product is 165 base
pairs, the PaxFL product is 237 base pairs, and the Pax5Δ2 product is 198 base pairs.

The Tubulin 60 product is 154 base pairs, the secreted product is 143 base pairs, and the membrane product is 177 base pairs.

Figure 13: Gel of qPCR products, showing that only one product is formed in each reaction.

LPS Activation to Determine if B-cell Activation Correlates with Usage of Pax5Δ2

SPL and PBL cells were freshly isolated from *O. mykiss* samples and cultured *in-vitro* in the presence of LPS. Cells were collected on days 0, 2, 4, and 7. Real-time PCR was performed on the samples using the primers discussed above. Figures 14 and 15 show scatter plots of the ratio of secreted/membrane IgM and the ratio of Pax5Δ2/Pax5FL for SPL and PBL respectively. These plots show a positive correlation between the levels of secreted IgM and Pax5Δ2. For this project, four sets of LPS activation were analyzed. The results from each set are quite different, but the following scatter plots illustrate the general pattern that was expected based on previous research. A large die-
off of cells between Day 0 and Day 2 occurred, thus the data points illustrated are for Day 2, Day 4, and Day 7. More LPS studies need to be completed to verify these results.

**Figure 14:** Scatter plot of the ratio of secreted/membrane IgM and the ratio of Pax5Δ2/Pax5FL for SPL LPS activation.

**Figure 15:** Scatter plot of the ratio of secreted/membrane IgM and the ratio of Pax5Δ2/Pax5FL for PBL LPS activation.

**Levels of IgM and Pax5 During Spawning**

To analyze how the levels of IgM and Pax5 change during spawning in *O. nerka*, we needed to find a suitable negative control for the spawning fish. Because it is very difficult to obtain fish from the open ocean, where levels of IgM and Pax5 would not yet have been subjected to the effects of spawning, an alternative negative control was
necessary. For this experiment, *O. mykiss* fish from the laboratory were used as a negative control.

Real-time PCR was run using the *O. mykiss* anterior kidney, posterior kidney, spleen and PBL samples and the IgM and Pax5 primers, as described above. These samples, which should not be exhibiting an immune response, show a positive correlation between secreted IgM and Pax5Δ2 levels, consistent with the results of the LPS experiment for SPL and PBL. The correlation is not seen in the anterior kidney due to the large number of B cells at different developmental and activation stages within this organ: anterior kidney is the site of both B-cell lymphopoeisis and storage of long-lived plasma cells. The presence of a large number of plasma cells, which are secreting large quantities of IgM, but have no Pax5, could have a strong influence on the data for this tissue. Additionally, at the current time, not enough is known about the maturational stages of B-cells in the posterior kidney to make analysis of these data beneficial.

**Figure 16:** *O. mykiss* SPL shows a strong positive correlation between Secreted IgM and Pax5Δ2.
Figure 17: *O. mykiss* PBL shows a positive correlation between Secreted IgM and Pax5Δ2.

Data from real-time PCR of all samples using the primers and methods described above was graphed in many different ways in order to determine the correlation between secreted IgM levels and Pax5Δ2 for the spawning *O. nerka* samples. The following figures show the ratio of Secreted/Membrane IgM for each individual fish, the ratio of Pax5Δ2/Pax5FL for each individual fish, and then the correlation between these two ratios. Due to small number of samples, random variation between samples has a large effect on the patterns generated by the data. The data are shown in the form of ratios in order to reduce the effects of these random variations between different samples of the population.

A difference in the ratio of secreted/membrane IgM and subsequently the ratio of Pax5Δ2/Pax5FL was expected between male and female fish based on the literature. After graphing the sexes separately, a pattern was not apparent (see Appendix). Sex is indicated in the following graphs.
The SPL and PBL of spawning *O. nerka* samples exhibit the same positive correlation between secreted IgM and Pax5Δ2 that was seen in our highly defined *O. mykiss* immune response generated through LPS activation (Figure 20 and Figure 23 respectively). A scatter plot showing the correlation between the secreted IgM and Pax5Δ2 is shown for AK and PK (Figure 26 and Figure 29). We see no correlation between IgM and Pax5 levels in these tissues due to the complex nature of the B-cell types found in the tissues (as described above).

**Figure 18:** SPL Ratio of Sec/Mem IgM. Sample location and sex indicated on graph. All *O. nerka* samples (numbered) had much higher levels of secreted IgM than *O. mykiss* (OM) negative controls. Ref refers to the sample used as a reference for the OM samples. OM samples were then used as the reference for the *O. nerka* analysis.
Figure 19: SPL Ratio of PaxΔ2/PaxFL. *O. nerka* samples 54, 55, 56, and 111 have very low levels of PaxFL. Samples from the Main Bay had levels of Pax5 similar to those seen in the *O. mykiss* (OM) samples.

Figure 20: Scatter plot comparing the ratio of Pax5Δ2/Pax5FL with the ratio of Sec/Mem IgM. $R^2$ value of 0.4267 indicates a positive correlation between secreted IgM and Pax5Δ2.
Figure 21: PBL Ratio of Sec/Mem IgM. All samples had very low levels of secreted IgM except for the two spawned males.

Figure 22: PBL Ratio of PaxΔ2/PaxFL. *O. mykiss* (OM) samples had higher levels of Pax5 than *O. nerka* samples except for the two spawned males.
Figure 23: Scatter plot showing the correlation between the ratio of Pax5Δ2/Pax5FL and Sec/Mem IgM. R² value of 0.7414 indicates a positive correlation between secreted IgM and Pax5Δ2.

Figure 24: AK Ratio of Sec/Mem IgM. Secreted IgM levels were higher in the *O. nerka* than in the *O. mykiss* (OM).
Figure 25: AK Ratio of Pax5Δ2/Pax5FL.

Figure 26: Scatter plot showing the correlation between the ratio of Pax5Δ2/FL and the ratio of Sec/Mem IgM. R^2 value of 0.1838 indicates no correlation between the data.
**Figure 27:** PK Ratio of Sec/Mem IgM. Spawning samples generally had higher levels of secreted IgM than the *O. mykiss* (OM) samples. Spawned fish reversed to lower levels of secreted IgM.

**Figure 28:** PK Ratio of PaxΔ2/PaxFL.
**Figure 29**: Scatter plot showing the correlation between the ratio of Pax5Δ2/Pax5FL and the ratio of Sec/Mem IgM in the PK. $R^2$ value of 0.1277 indicates no correlation between the data.
Discussion

The main goal of this project was to determine if there is a correlation between the alternative splicing of Pax5 and an immune response in *Onchorynchus* spp. Here we showed that the level of Pax5Δ2 positively correlates with the levels of secreted IgM *in-vitro* in LPS activated *O. mykiss* B-cell, *in-vivo* in *O. mykiss* samples, and *in-vivo* in *O. nerka* samples. Pax5 has previously been shown to be alternatively spliced in mouse B-cells (Zwollo et al., 1997). Alternatively spliced isoforms are thought to function through a number of different mechanisms including: changes in DNA binding properties, differential interaction with co-activators or co-repressors, or through changes in interaction with the transcription initiation complex (Zwollo, 2011). Previous research in the Zwollo lab has shown that mouse B cells, in addition to expressing the full-length form of Pax5, Pax5FL, express at least three different isoforms. One of these isoforms, isoform Pax-5b (Pax5Δ2) has a deletion of exon 2, resulting in a Pax5 protein with a very short paired domain that is unable to bind DNA *in-vitro* (Zwollo et al., 1997). Our lab subsequently showed that *O.mykiss* also expresses this isoform in B cells.

**IgM Changes During Spawning (Humoral Immune Response during Spawning)**

From the data presented here, it appears that during spawning, there is a relative increase in the levels of secreted IgM compared to the levels of membrane IgM. However, the absolute levels of secreted IgM were decreased in spawning fish. These results make sense when one considers what happens to the fish as it moves towards the spawning grounds. First, the fish will need some sort of immune activity to fight
pathogens encountered during the trip. Cortisol levels, which are highly increased due to
the spawning-induced stress response, will have a profound immunosuppressive effect on
the fish. All developing B cells are lost through the inhibition of B-lymphopoiesis. In
addition, pre-B cells, activated B-cells and possibly plasmablasts are lost through
apoptosis, but plasma cells and mature B-cells do not appear to be strongly affected by
cortisol (Harris and Bird, 2000). Thus, there is a decrease in absolute levels of both
membrane and secreted IgM due to the effects of cortisol, but a relative increase in
secreted IgM, because cortisol does not appear to be affecting the plasma cells directly.

Before beginning this project, it was hypothesized that male and female samples
would have different patterns of expression for relative IgM levels and, in turn, relative
Pax5 levels. The rationale for this hypothesis was that the female immune system would
have to be more strongly activated in order to provide and pass on Ig-protection to the
eggs (Swain and Nayak, 2009). Maternal immunity transmission is defined as the
transfer of immune factors from an immunocompetent female to an immunologically
naïve neonate through yolk. Successful transfer of maternal IgM to eggs and embryos
has been documented in several teleostean fishes including the rainbow trout. In fish,
both innate and adaptive immunity are transferred from mother to offspring through
factors including immunoglobulin, complement factors, lysozymes, protease inhibitors,
lectins, and serine protease like molecules. Maternally derived IgM persists for a limited
time, exhausts around completion of the yolk absorption process, and completely
disappears during larval stages. Maternal transfer of immunity depends on the health and
immune status of the brood fish. For fish embryos and larvae to survive against
microbial infection maternal immunity is required. The mechanism of IgM uptake and
storage within eggs is unknown. Maternally transferred IgM provide defense against infectious agents (Swain and Nayak, 2009). Thus, based on the fact that IgM must be transferred from mother to egg during spawning, it was hypothesized that the female immune system (in terms of the levels of secreted and membrane IgM) would look different from the male immune system at that time.

It is well known that sex steroids have an effect on the immune system. Thus, the hypothesis that the male and female immune systems would look different during spawning was because different hormones have differing effects on the immune system and males and females produce different hormones. In *O. mykiss* estrogen is shown to stimulate the proliferation of PBL *in vitro*. The rise of sex hormones such as estradiol-17β, oestradiol, testosterone, 11-ketotestosterone and androstenedione can suppress the plasma IgM and IgM secreting cells during spawning period leading to immunosuppressive condition in fish (Harris and Bird, 2000 and Swain and Nayak, 2009). In mammals, androgens are associated with male and estrogens with female reproductive function. In fish, testosterone and androstenedione are major products in female fish. In the teleost testis, 11-ketotestosterone of 11b-OH testosterone are the most potent androgens (Harris and Bird, 2000). Based on these data, it appears that the male immune system, and IgM levels, are more strongly affected by sex steroids. Both male and female sexually mature salmonids have immune deficiencies during spawning and are subject to increased ectoparasitic infestations (Harris and Bird, 2000). Thus, based on this hypothesis presented here, it would follow that the immune systems of males and females, while both deficient during spawning, would be differently affected by the hormones that they are secreting at this time.
Male and female samples did not appear to have strong differences in IgM relative expression levels nor in Pax5 relative expression levels in this study. More samples would need to be collected and analyzed in order to strengthen this conclusion.

**Pax Changes During Humoral Immune Response**

Our data support the model that the level of Pax5Δ2 (relative to Pax5FL) increases as the level of secreted IgM (relative to membrane IgM) increases in *Oncorhyncus* immune tissues. This pattern of expression was strongly supported by freshly isolated samples from *O. Mykiss* that had not experienced any form of laboratory induced stress or immune system activation. These samples, from fish who were kept as healthy as possible in the laboratory, showed a strongly positive linear correlation ($R^2 = .99$) between secreted IgM and Pax5Δ2 levels in the spleen. PBL samples also showed a positive linear correlation, but with a less strong coefficient of linearity ($R^2 = 0.59$). Our LPS activated *O. mykiss* samples also weakly exhibited a positive correlation between secreted IgM and Pax5Δ2. The Pax5Δ2 expression relative to Pax5FL appears to increase as the relative levels of secreted IgM increase both when the immune system is not activated and when it is activated. From the study of spawning *O. nerka*, our data indicate that as the immune system activates in order to fight pathogens encountered during spawning, there is also an increase in relative levels of secreted IgM and this increase correlates with an increase in the relative levels of Pax5Δ2. These results suggest that Pax5Δ2 may be involved in the alternative splicing of the IgM gene to switch from membrane to secreted forms of the protein.
However, it has been shown by others that LPS-induced Blimp1 plays a direct role in the switch from membrane to secreted IgM (Kallies and Nutt, 2007). Blimp1 suppresses Pax5 expression upon B cell activation, but in this present study an increase in the levels of Pax5Δ2 was seen as more secreted IgM was produced. It is possible that as B cells become more activated, they start making more Pax5Δ2, which acts as a “temporary, dominant negative” form of the Pax5FL protein as a means to lower Pax5 function, but not have it fully repressed. Ultimately, as a B cell becomes a plasma cell, Pax5 levels will become completely repressed. In-between states of terminal B cell differentiation may use alternative splicing of Pax5 to regulate its own function and thus allow proper expression level of the transcription factor until the plasma cell stage where Pax5 is no longer needed.

**Directions for Future Studies**

This study provided support for the idea that Pax5Δ2 isoform expression relative to Pax5FL expression increases as the level of secreted IgM increases relative to the level of membrane IgM. The data generated from the anterior and posterior kidney were difficult to interpret based on the large variety of cell type found in these tissues. Both tissues are expected to contain developing B cells (which have high levels of Pax5), and several antibody-secreting cell types, including plasmablasts and plasma cells (Zwollo et al., 2010). Therefore, qPCR is not the preferred method of analysis for these tissues, Flow Cytometric analysis of all the different stages of B-cell found in the spawning fish would enable us to further understand the data obtained from these tissues.
Additionally, it would be very interesting to perform a functional study of the Pax5Δ2 isoform in these samples. A functional study would determine if our hypothesis that Pax5Δ2 is acting as a co-repressor of Pax5FL and allowing activated mature B-cells to move toward becoming plasma cells that secrete IgM is correct. Techniques such as transient transfections, eletrophoretic mobility shift assays, and many others would be used for functional analysis of the Pax5Δ2 isoform. This research is significant because Pax5 has been implicated in playing a role in multiple forms of B-cell lymphomas as well as other forms of cancer in humans (Decker et al., 2008). Therefore, this research could provide important insights into the role that Pax5Δ2 plays in oncogenesis or in the maintenance of the oncogenic phenotype. Additionally, a functional analysis could provide insight on how Pax5Δ2 functions during B-cell maturation and the switch from plasmablast to IgM secreting plasma cell. Determining the expression pattern and function of Pax5 isoforms is an important first step in fully characterizing the B-cell.

To strengthen the real-time PCR results, a number of experiments need to be conducted. First, we will need to repeat in vitro LPS activation experiments in *O.mykiss*, or even better, *O. nerka*, to obtain more consistent results. Our current data from four LPS activations were not consistent enough to fully illustrate what is happening during activation. From the LPS data shown in the results section, it is apparent that there is a positive correlation between secreted IgM levels and Pax5Δ2; however, the cells in this study experienced a massive die-off between day 0 and day 2, and then never appeared to become strongly activated. Because this part of the study is being used as an in vitro control, it is very important that this control be repeated and results more strongly supported. Additionally, we need to obtain *O. nerka* samples that are not from spawning
fish to use as a proper reference sample, and also to understand and appreciate better the changes that occur during spawning. Although unlikely based on the highly conserved nature of the Pax5 gene, the *O. mykiss* samples could potentially differ significantly from the *O. nerka*. Thus, using the rainbow trout as a reference sample may be affecting the results. Finally, more samples need to be analyzed. Currently, the number of samples analyzed for this experiment from each location is very small which could lead to skewed results. Collecting and analyzing at least 10-15 total animals per site, will help to standardize our relative expression levels and will strengthen our results.

Season and environment have also been shown to affect the immune system of brood fish. Thus, measurement of the water temperature, pH, and O₂ content where samples are being collected would also be interesting variables to include. It is established that seasonal cycles affect several biological activities in fish (summarized in Swain and Nayak, 2009). Among the environmental factors, temperature is known to affect the fish immune system. IgM levels are reduced when the temperature is very low. Conversely, serum protein levels, including IgM, rise during the summer in several species of fish (Swain and Nayak, 2009). From these data, it is apparent that water temperature and other variables can strongly affect the immune system of fish, thus it would be interesting to include water quality variables in our study.

This project has provided insight into the relationship between the alternative splicing of Pax5 and an immune response in spawning fish. More work needs to be done to strengthen the results of this study, but this study has optimized the assays needed to analyze future samples and thus has laid the framework for the future study of this system.
RNA Quality

The quality of RNA used in a qPCR reaction needs to be very high because the accuracy of gene expression studies is influenced by the quantity and quality of the starting RNA. Low quality RNA can strongly compromise the results of downstream applications (Fleige and Pfaff, 2006). RNA samples that contain genomic DNA or are degraded will cause the SYBR green to bind non-specifically and can drastically affect results. The first major obstacle in for this project was obtaining high quality RNA. High quality RNA is defined as RNA that is free of protein (260/280nm greater than 1.8), free of genomic DNA, undegraded (28S:18S ratio should be between 1.8 and 2.0), free of enzymatic inhibitors, free of substances which complex with reaction co-factors (Mg$^{2+}$ or Mn$^{2+}$), and free of nucleases for storage (Fleige and Pfaffl, 2006). Tissues such as the spleen and anterior kidney contain a large number of enzymes, making the process of extracting RNA from these tissues very difficult. Before starting the real-time PCR assay optimization, a RNA extraction protocol needs to be optimized in order to obtain a sufficient quantity of high quality RNA from the precious samples collected in Alaska. The Trizol protocol described in the Methods allowed us to obtain high quality RNA based on spectrophotometric analysis using the NanoDrop.

In this study, the RNA from the 2010 samples is of slightly higher quality (based on 260/280 ratios greater than 1.8) than the RNA from the 2009 fish collection. After discovering the difficult nature of RNA extraction from these tissues, more precaution
was taken when storing samples from the 2010 collection. These precautionary steps included slicing the tissue to allow RNAlater access to more parts of the tissue and placing the sample on ice immediately to slow enzymatic degradation of the RNA.

**Importance of Careful Assay Design**

PCR is the most sensitive method available to discriminate between closely related mRNAs. Real-time PCR is highly sensitive and is able to quantify a range of up to seven orders of magnitude (Jain et al., 2006). Thus, we chose real-time PCR as our method of choice for studying differential expression of Pax5 isoforms in B-cells. In order for this experiment to produce reliable data, the efficiency of the real-time PCR design was critical. The use of SYBR green for the real-time PCR required extensive optimization to ensure that the fluorescence measured was the result of specific product and not non-specific binding (Dorak, 2011). Primers were carefully designed to: 1) anneal specifically to the desired sequence, 2) to produce only one product, and 3) to avoid SYBR green dye binding to non-target, double-stranded DNA. Additionally, comparative real-time PCR results must be normalized with a reference or housekeeping gene. This gene should remain constant between cells of different tissues and under different environmental conditions. If the expression of this gene changes between tissues or due to differing environmental changes, it could lead to erroneous results (Jain et al., 2006). For this experiment, tubulin was chosen as the internal control for gene expression analysis because of its uniform expression. Thus, all calculations in this study were normalized to the level of tubulin found in each sample.
We used melt curve analysis to prove that the assay was performing optimally during each real-time run. During real-time PCR the instrument monitors both DNA synthesis as well as the melting point of the product at the end of the amplification reactions. The melting temperature depends on both the length and base composition of the DNA double helix. An assay is running optimally when all PCR products for a particular primer pair have the same melting temperature. Primer-dimer artifacts give a peak with a lower melting temperature due to the short DNA sequence (Hunt, 2006).

The Use of the $\Delta\Delta C_T$ Method for qPCR Analysis

Data from real-time, quantitative PCR experiments can be analyzed via absolute quantification and relative quantification. Relative quantification relates the relative PCR signal of the target transcript to that of a control (sample at day 0, or $O.\ mykiss$ sample in this study). In this study we used relative quantification via the $\Delta\Delta C_T$ method. The $\Delta\Delta C_T$ method is an effective method for analyzing relative changes in gene expression from real-time PCR studies (Livak and Schmittgen, 2001). For the $\Delta\Delta C_T$ method to be valid, a validation experiment must be performed to verify that the target and reference reaction efficiencies are similar (Livak and Schmittgen, 2001). The validation experiment was performed by making a series of dilutions of primer and verifying that they have similar efficiency (see Validation Experiment in Results). The internal control gene and reference samples were chosen carefully because the values generated by these samples will be used as a calibrator for the $\Delta\Delta C_T$ calculations (Livak and Schmittgen, 2001). Choosing a reference sample was difficult in this study because it is very difficult to obtain samples from non-spawning, adult $O.\ nerka$, which reside in the open ocean. Thus, $O.\ mykiss$ samples from laboratory tanks were used to model the
secreted/membrane IgM and Pax5 transcript levels that may be found in *O. nerka* samples before beginning their journey to the spawning grounds. Once these important variables had been chosen and measured, data were analyzed using the ΔΔCT method (see Methods for Equations).
Appendix

*O. mykiss* Spleen LPS Data

**Figure 14A:** Fold change secreted IgM

**Figure 14B:** Fold change membrane IgM
**Figure 14B:** Fold change membrane IgM

**Figure 14C:** Fold change Pax5FL
**Figure 14D:** Fold change Pax5Δ2

**Figure 14E:** Fold change secreted and membrane IgM
Figure 14F: Ratio of Sec/Mem IgM

Figure 14G: Fold change Pax5FL and Pax5Δ2.
**Figure 14H:** Ratio of Pax5Δ2/Pax5FL Values for Pax5Δ2 are very low.

**Figure 14I:** Ratio of Pax5FL/Pax5Δ2
Figure 14J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5Δ2/Pax5FL fold changes.

\[ y = 0.1803x + 0.8791 \]
\[ R^2 = 0.11781 \]

O. mykiss Blood LPS Data

Figure 15A: Fold change secreted IgM
**Figure 15B:** Fold change membrane IgM

**Figure 15C:** Fold change Pax5FL
**Figure 15D:** Fold change Pax5Δ2

**Figure 15E:** Fold change secreted and membrane IgM.

**Figure 15F:** Ratio of Sec/Mem IgM
**Figure 15G:** Fold Change Pax5FL and Pax5Δ2.

**Figure 15H:** Ratio of Pax5Δ2/Pax5FL
**Figure 15I:** Ratio of Pax5FL/Pax5Δ2

**Figure 15J:** Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5Δ2/Pax5FL fold changes.

**Spleen 2009 Data**

**Figure 16A:** Fold change secreted IgM
Figure 16B: Fold change membrane IgM

Figure 16C: Fold change Pax5FL

Figure 16D: Fold change Pax5Δ2. * SPL 54 and SPL 69 removed due to exceptionally high fold change values.
**Figure 16E:** Fold change secreted and membrane IgM.

**Figure 16F:** Ratio of Sec/Mem IgM
Figure 16G: Fold change Pax5FL and Pax5Δ2

Figure 16H: Ratio of delta2/FL

Figure 16I: Ratio of Pax5FL/Pax5Δ2
**Figure 16J:** Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes.

**Anterior Kidney 2009 Data**

**Figure 17A:** Fold change secreted IgM
Figure 17B: Fold change membrane IgM

Figure 17C: Fold change Pax5FL

Figure 17D: Fold change Pax5Δ2
**Figure 17E:** Fold change secreted and membrane IgM

**Figure 17F:** Ratio of Sec/Mem IgM
**Figure 17G:** Fold change Pax5FL and Pax5Δ2

**Figure 17H:** Ratio of Pax5Δ2/Pax5FL

**Figure 17I:** Ratio of Pax5FL/Pax5Δ2
Figure 17J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes.

Posterior Kidney 2009 Data

Figure 18A: Fold Change Secreted IgM.

Figure 18B: Fold change membrane IgM.
**Figure 18C:** Fold change Pax5FL.

**Figure 18D:** Fold change Pax5Δ2. *Sample PK 68 removed because the fold change value was 22.41 which made the other samples appear to have no Pax5Δ2.

**Figure 18E:** Fold change for Sec and Mem IgM together.
**Figure 18F:** Ratio of Sec/Mem IgM.

**Figure 18G:** Fold Change Pax5FL and Pax5Δ2. Sample 68 was left out of this graph because the fold change value was very high and made the other samples appear to have negligible amounts of Pax5.

**Figure 18H:** Ratio of Pax5Δ2/Pax5FL.
Figure 18I: Ratio of Pax5FL/Pax5Δ2.

Figure 18J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes (outliers have been removed).

Blood 2009 Data

Figure 19A: Fold change secreted IgM
**Figure 19B**: Fold change membrane IgM

**Figure 19C**: Fold change Pax5FL

**Figure 19D**: Fold change Pax5Δ2
**Figure 19E:** Fold change secreted and membrane IgM

**Figure 19F:** Ratio of Sec/Mem IgM

**Figure 19G:** Fold change Pax5FL and Pax5Δ
Figure 19H: Ratio of Pax5Δ2/Pax5FL

Figure 19I: Ratio of Pax5FL/Pax5Δ2

Figure 19J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes.
Spleen 2010 Data

Figure 20A: Fold change secreted IgM

Figure 20B: Fold change membrane IgM
Figure 20C: Fold change Pax5FL

Figure 20D: Fold change Pax5Δ2
**Figure 20E**: Fold change secreted and membrane IgM

**Figure 20F**: Ratio of Sec/Mem IgM
Figure 20G: Fold change Pax5FL and Pax5Δ2

Figure 20H: Ratio of Pax5Δ2/Pax5FL

Figure 20I: Ratio of Pax5FL/Pax5Δ2
**Figure 20J:** Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes.

**Anterior Kidney 2010 Data**

**Figure 21A:** Fold change secreted IgM

**Figure 21B:** Fold change membrane IgM
Figure 21C: Fold change Pax5FL

Figure 21D: Fold change Pax5Δ2

Figure 21E: Fold change secreted and membrane IgM.
**Figure 21F:** Ratio of Sec/Mem IgM

**Figure 21G:** Fold change Pax5FL and Pax5Δ2

**Figure 21H:** Ratio of Pax5Δ2/Pax5FL
Figure 21I: Ratio of Pax5FL/Pax5Δ2

Figure 21J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes.
Posterior Kidney 2010 Data

**Figure 22A:** Fold change secreted IgM.

**Figure 22B:** Fold change membrane IgM. *Samples 111 and 112 have been removed from the analysis due to very high membrane IgM levels.
Figure 22C: Fold change Pax5FL.

Figure 22D: Fold change Pax5Δ2.
**Figure 22E:** Fold change secreted and membrane IgM together. *Samples 111 and 112 have been removed due to extremely high membrane IgM values.*

**Figure 22F:** Ratio of Sec/Mem IgM.
Figure 22G: Fold change Pax5FL and Pax5Δ2 together.

Figure 22H: Ratio of Pax5Δ2/Pax5FL.

Figure 22I: Ratio of Pax5FL/Pax5Δ2.
Figure 22J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax5Δ2 fold changes.

Blood 2010 Data

The PBL 2010 yielded very poor quality RNA and thus only five samples were seen as fit to use in qPCR.

Figure 23A: Fold change secreted IgM

Figure 23B: Fold change membrane IgM
Figure 23C: Fold change Pax5FL

Figure 23D: Fold change Pax5Δ2

Figure 23E: Fold change secreted and membrane IgM
Figure 23F: Ratio of Sec/Mem IgM

Figure 23G: Fold change Pax5FL and Pax5Δ2

Figure 23H: Ratio of Pax5Δ2/Pax5FL Fold Change
Figure 23I: Ratio of Pax5FL/Pax5Δ2

Figure 23J: Scatterplot comparing the Ratio of Sec/Mem IgM Fold Changes with the Ratio of Pax5FL/Pax52 fold changes.

Spleen
Figure 24: Ratio of Sec/Mem IgM for male and female samples.

Figure 25: Ratio of Pax5Δ2/Pax5FL for male and female samples.
Anterior Kidney

Figure 26: Ratio of Sec/Mem IgM for male and female samples.

Male AK Ratio of Sec/Mem IgM

Female AK Ratio of Sec/Mem IgM

Male AK Ratio of Pax5Δ2/Pax5FL
Figure 27: Ratio of Pax5Δ2/Pax5FL for male and female samples.

Posterior Kidney

Figure 28: Ratio of Sec/Mem IgM for male and female samples.
Figure 29: Ratio of Pax5\(\Delta 2/Pax5FL\) for male and female samples.

PBL
Figure 30: Ratio of Sec/Mem IgM for male and female samples.

Figure 31: Ratio of Pax5Δ2/Pax5FL for male and female samples.
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