ADVANCING THREESPINE STICKLEBACK FISH AS A

NOVEL IMMUNOGENETICS MODEL BY PINPOINTING

THE ONSET OF ADAPTIVE IMMUNITY

by

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Understanding the onset of the adaptive immune system is important for understanding host-microbe interactions and the development of disease phenotypes. While the onset of adaptive immunity has been previously studied in model organisms such as mice and zebrafish, these inbred laboratory models are challenged by a lack of genetic diversity and may not be appropriate for all immunological studies. We advance threespine stickleback fish (*Gasterosteus aculeatus*) as an outbred immunogenetics model in order to study the onset of the adaptive immune system in the context of genetic variation. Threespine stickleback fish exist in various coastal habitats throughout the Northern hemisphere and exhibit natural genetic diversity within families and between populations. Although this teleost model has been effective in previous immunological studies, there are foundational questions still left unanswered including, when does the onset of the adaptive immune system occur in threespine stickleback? To pinpoint the onset of adaptive immunity, we looked at two populations of threespine stickleback over a developmental time series and analyzed the expression of a gene involved in the development of T-lymphocytes. T-lymphocytes are a primary adaptive immune cell type able to recognize and elicit a response against pathogens.

Early development of these cells involves the TCR/CD3 protein complex composed of six subunits that are necessary for proper T-cell receptor (TCR) expression and cell activation in mature T-lymphocytes. Genes encoding the TCR/CD3 complex have been previously used to study the ontogeny of immune cells and have provided important insights into the development of the adaptive immune system. In this study, we chose to focus on *cd3d*, a gene encoding one subunit of the TCR/CD3 complex. Similar work determining the timing of onset of adaptive immunity in other fish species has produced a wide range of results, from 11 hours post fertilization to 26 days post hatching (dph). We found that by 10 dph, *cd3d* was expressed in all individuals, with population level variation indicating some individuals may exhibit expression earlier in development.

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Chapter 1

Introduction to Genetics

Scientists have long been interested in heredity and the variation of genetic and physical traits. Heredity can be explained by the fundamental laws of inheritance, first discovered by 'the father of genetics' Gregor Mendel through his work on pea plants (Mendel 1866). Mendel was able to determine that genetic traits are inherited as distinct units, as opposed to the previous theory of blending inheritance; there are dominant and recessive traits passed from parents to offspring; and traits are passed on independently of other traits (Mendel 1866). Within years of Mendel's work, Charles Darwin, the 'father of evolution', proposed the theory of biological evolution by natural selection explaining how species change over time as result of heritable traits; changes that are beneficial for an organism are maintained through 'survival of the fittest' in an environment (Darwin 1859). However, Darwin's theory was lacking a mechanism for inheritance, so his theory was incomplete alone. The modern synthesis fuses Mendelian genetics with Darwinian evolution allowing for modern evolutionary biology to understand inheritance and variations in heritable traits (Huxley 1942). While Mendel's and Darwin's work contributed to our understanding of how phenotypes – observable characteristics – are inherited and selected for, they did not know about DNA or genes. Nearly a century later, Watson and Crick discovered the double-helix structure of DNA, giving rise to modern molecular biology (Watson & Crick 1953).

The central dogma of genetics explains the flow of genetic information from the expression of a genotype to the expression of a phenotype, flowing from DNA to RNA to protein. Deoxyribonucleic acid (DNA) is a double-stranded molecule that carries

genetic information for the development, function, growth, and reproduction of organisms. DNA contains genes, the basic physical and functional units of heredity, that can be transcribed into another molecule to carry genetic information. The initiation of gene transcription occurs when RNA polymerase binds to the promoter region of a gene. Then, the DNA double helix is unwound and the strands are broken apart so that one strand can be read as a template to create a single-stranded, complementary messenger ribonucleic acid (mRNA) molecule. mRNA acts as the genetic messenger, carrying instructions for protein synthesis from DNA to the ribosome. The nucleotides composing the mRNA molecule are 'read' in triplets, or codons, each corresponding to an amino acid. By this process, the RNA undergoes translation and is decoded to form a specific chain of amino acids known as a protein. This entire flow of genetic information, as well as other regulating factors, can lead to the expression of genes in an individual.

While heritable genotypes are a major cause of intraspecific variation, environmental factors and genetic background can also influence the variation of phenotypes. Studies in a variety of animal models have revealed that the environment has a strong influence on the development of the immune system. For example, there is differential expression of immune parameters between wild-caught and lab-reared mice *(Mus musculus domesticus*) and differential immune gene expression between lake and river populations of threespine stickleback fish (*Gasterosteus aculeatus*) (Huang *et al.* 2016; Abolins *et al.* 2017). Environmental stress factors such as oxygen levels, pollutant exposure, and pathogens can create developmental and long-term changes to the immune system causing the organism to be more susceptible to disease and

infection (Bowden 2008). In addition to environmental factors, the organism's genetic background can be important for the variable expression of phenotypes between individuals – even those with the same genotype. Genetic background is the combination of genes that may interact with a gene of interest, and potentially alter the expressed phenotype (Linder 2006). Genetic background is especially important in the development and progression of disease states (Rosas *et al.* 2005; Spagnuolo *et al.* 2016). For example, patients with the genetic mutation causing cystic fibrosis (CF) are more likely to have severe, chronic pulmonary infections of the bacterium *Pseudomonas aeruginosa* than patients with other pulmonary diseases such as chronic obstructive pulmonary disease (COPD) (Spagnuolo *et al.* 2016). While some interactions between genetics and the immune system, such as the CF example, are understood, there is still much we don't understand about immunogenetics.

The Immune System

Previously, the immune system was understood primarily as an organism's defense against pathogens and infection, but ongoing research has revealed that the immune system has many other responsibilities (Delves & Roitt 2000). For example, it maintains homeostasis between bacteria and the host and works closely with many other body systems such as the circulatory and lymphatic systems for cell production and transportation (Delves & Roitt 2000; Hooper *et al.* 2012; Cueni & Detmar 2013; Girard *et al.* 2012). Additionally, the immune system can cause autoimmune disorders when it doesn't function properly by attacking the host instead of fighting against infection (Delves & Roitt 2000; Hooper *et al.* 2012).

The vertebrate immune system is characterized by two branches: the innate immune system and the adaptive immune system. Both branches, composed of specialized cells and molecules, are designed to respond to microbes and prevent infection (Delves & Roitt 2000). Individually, the innate immune system is able to respond to extracellular pathogens but can also work in conjunction with the adaptive immune system to eliminate intracellular pathogens (Delves & Roitt 2000). Although the two branches can work in conjunction, there are many key differences between them, particularly in the development, response time and physiology of each.

The innate immune system serves as the first line of defense against pathogens, and begins to develop early in embryogenesis, as early as in embryonic stem cells (Guo 2019). Innate immunity involves a variety of cells that are able to mount a quick immune response and are thought to be especially important in targeting viral and bacterial pathogens at mucosal surfaces (Hamerman *et al.* 2005). This system is composed of inflammatory cells, phagocytic cells, and natural killer cells. Additionally, innate responses often involve the complement system, acute-phase proteins and cytokines in order to notify the immune system of infection, respond to the infection, and clear out the pathogen (Delves & Roitt 2000).

The various cell types of the innate immune system each have unique functions in response to pathogens. Inflammatory cells release cytokines and proteins in order to promote inflammation; this inflammation can protect the infected tissue from further damage by pathogens (Abdulkhaleq *et al.* 2018). There is a collection of cells that can induce inflammation: macrophages, neutrophils and lymphocytes. Macrophages are able to perform endocytosis by engulfing foreign particles, bacteria, and infected cells

to destroy them and remove the pathogens (Hirayama *et al.* 2018). As inflammatory

cells, macrophages can also release inflammatory cytokines, acute phase proteins and

antimicrobial peptides to aid in the immune response (Hirayama *et al.* 2018). Natural

killer (NK) cells, rather than directly clearing the infection, initiate cytolytic activity

against infected cells (Paul & Lal 2017). This activity includes releasing cytokines and

chemokines to modulate the function of other immune cells that can destroy the

pathogen (Paul & Lal 2017). NK cells, as part of the innate immune system, mount a

quick immune response and are thought to be especially important in targeting tumors

and virus-infected cells (Hamerman *et al.* 2005).

  With nonspecific immune cells, the innate immune system is able to recognize

microbes by germline encoded pattern recognition receptors (PRRs) that recognize

common pathogen-associated molecular patterns (PAMPs) such as peptidoglycans and

lipopolysaccharides (LPS) in bacterial cell walls or viral double-stranded RNA

(Medzhitov & Janeway 2000). Germline encoded PRRs are limited in the number of

PAMPs that they can recognize because the receptor repertoire is established by the

germline and does not change over the host's lifetime (Medzhitov & Janeway 2000;

Magnadóttir 2005). The responses elicited by the innate immune system are nonspecific

and mainly involve phagocytic and cytotoxic cells that can rapidly respond to an

infection by releasing molecules such as antimicrobial peptides and inflammatory

cytokines (Janeway 1989; Magnadóttir 2005). The innate immune system, as the first

line of defense against pathogens, is vital for protecting the host until a more specific

immune response can be mounted.

The adaptive immune system, in comparison, mounts more specific immune responses than the innate immune system. Specific immune responses are primarily carried out by lymphocytes, including B-lymphocytes that mature in the bone marrow and T-lymphocytes that mature in the thymus. The development of immune cells in the bone marrow makes this branch of the immune system unique to the subphylum Vertebrata (Hirano *et al.* 2011; Zhao & Elson 2018). Additionally, the onset of the adaptive immune system may occur as early as embryogenesis, but the specific timing of onset is generally unclear. Attempts to study the onset of adaptive immunity have utilized early adaptive immune gene markers relating to the progenitors of T- and B-lymphocytes (Willett *et al.* 1997; Seemann *et al.* 2017). Although, the definition of 'onset' and the methods used to determine the timing of onset have not been standardized, so there is a wide range of timepoints reported (Willett *et al.* 1999; Tian *et al.* 2009; Uribe *et al.* 2011).

The adaptive immune system contains B-lymphocytes, or B-cells, which are activated by the binding of antigen to a membrane-bound immunoglobin (T-cell independent activation) or by an interaction with another immune cell (T-cell dependent activation) (Bonilla & Oettgen 2010). In T-cell independent activation, the B-cell immunoglobin receptors recognize repeating molecular patterns on microbial surfaces and the cell becomes activated (Bonilla & Oettgen 2010). However, this form of activation can only partially activate the B-cell and the cell requires additional signals to become fully activated (Bonilla & Oettgen 2010). In contrast, T-dependent activation requires the interaction of a B-cell and a T-cell to be fully activated. In this type of activation, the B-cell serves as an antigen presenting cell (APC) to the T-cell (Bonilla &

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Oettgen 2010). When the B-cell presents an antigen peptide to the T-cell, there is direct

cellular contact and release of co-stimulating molecules from the T-cell that fully

activates the B-cell (Bonilla & Oettgen 2010). Once the B-cell is activated, it is able to

proliferate and differentiate into its effector form, a plasma cell. The plasma cell

undergoes clonal expansion and is able to widely secrete antibodies specific to the

recognized antigen (Delves & Roitt 2000; Janeway & Medzhitov 2002). Secreted

antibodies tag the specific extracellular pathogen by binding to the surface and elicit an

immune response by recruiting phagocytes and other immune molecules (Janeway *et al*.

2001).

The adaptive immune system also contains T-lymphocytes, or T-cells, which

mature in the thymus and are activated when an antigen is recognized by the T-cell

receptor (TCR). The antigen is presented to the TCR by the major histocompatibility

complex (MHC) on an antigen presenting cell (Figure 2). The TCR is able to recognize

both the antigen being presented and the MHC (Kronenberg *et al.* 1986). To become

activated, the T-cell relies on the TCR/CD3 complex. The TCR heterodimer of the

complex recognizes the antigen peptide, then the CD3 subunits, with their longer

intracellular domains, are able to transduce the signal across the membrane to begin T-

cell activation (Clevers *et al.* 1988). The activated T-cell can then eradicate intracellular

pathogens by recruiting phagocytic or cytotoxic cells (Delves & Roitt 2000; Janeway &

Medzhitov 2002).
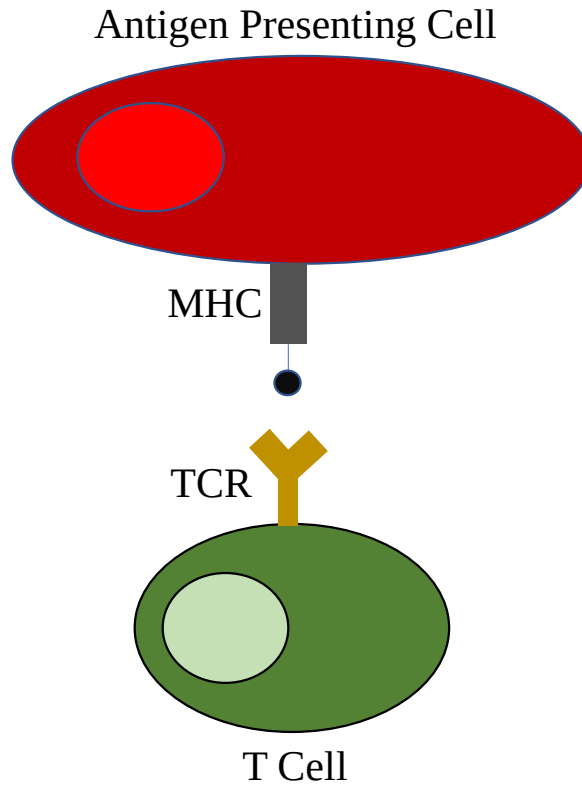
Figure 1: Interaction of the antigen presenting cell and the T-cell. The red antigen presenting cell has a surface major histocompatibility complex, represented as a grey projection, presenting an antigen peptide (black circle). The green T cell has a surface T cell receptor in yellow that recognizes the presented antigen.
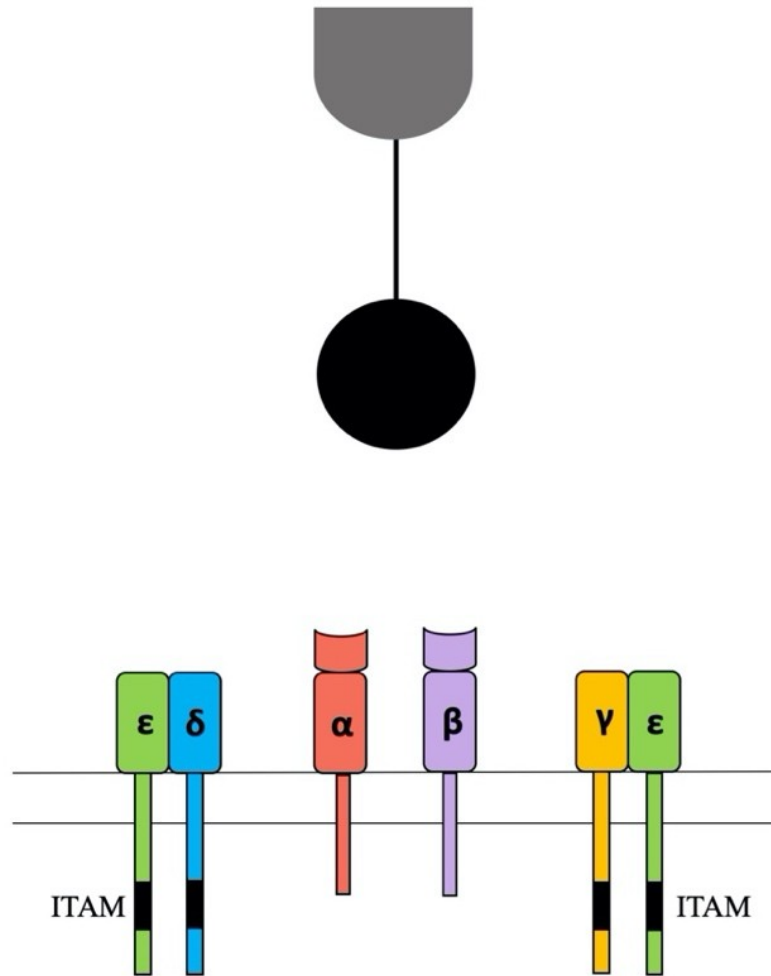
Figure 2: Interaction of antigen peptide with the TCR/CD3 complex. The TCR/CD3 complex is shown with each colored rectangle representing a chain of the complex. The Greek letter label the chains: TCRα (red), TCRß (purple), CD3δ (blue), CD3γ (yellow) and CD3ε (green; appears twice). The black boxes on the cytoplasmic tails on the CD3 components represent the ITAMS. An antigen (black circle) is being presented to the antigen recognition site (curved boxes on top of TCRα and TCRß) by the MHC (grey). Adapted from Franco et al. 2016.

T- and B-lymphocyte receptors, unlike innate immune receptors, are not germline-encoded and instead undergo V(D)J recombination in order to create a diverse repertoire of antigen-specific receptors. V(D)J recombination rearranges the variable (V), diversity (D) and joining (J) gene segments in the genomic DNA in order to form

the variable domain of T- and B-lymphocyte antigen receptors (Figure 1) (Mansilla-Soto & Cortes 2003). The V, D, and J gene segments are separated from each other in the DNA sequence of germline cells, so recombination brings the segments together to be expressed in mature lymphocytes (Janeway *et al.* 2001).
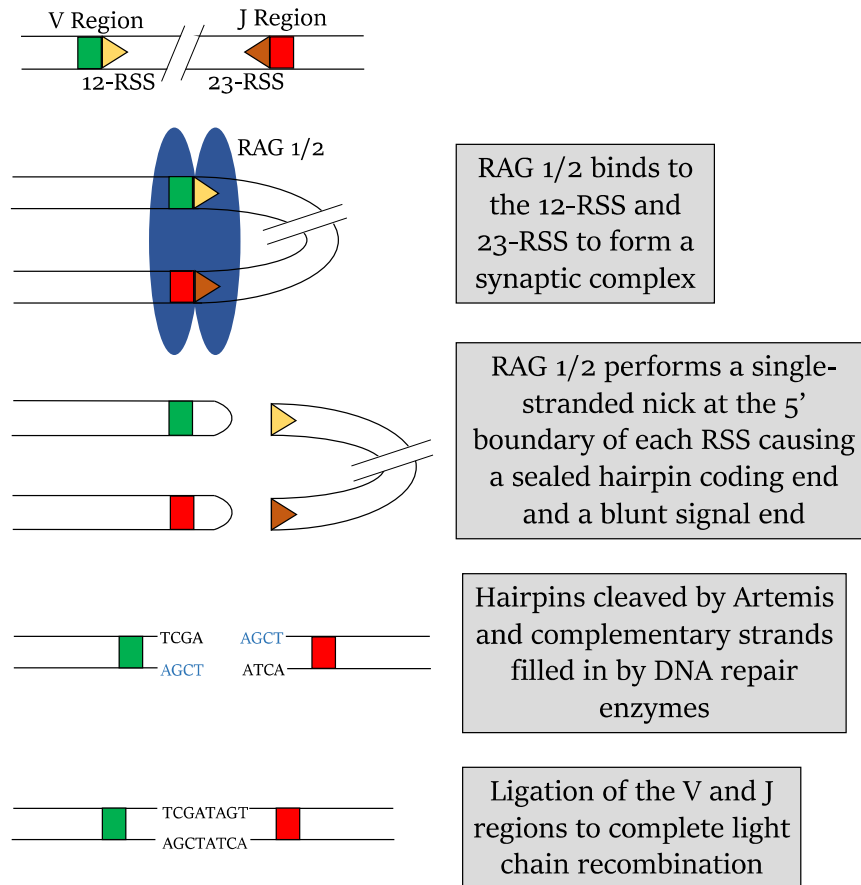
Figure 3: V(D)J recombination. Schematic representation of the recombination of a variety (V) and joint (J) gene segment. The blue ovals represent the RAG1/RAG2 protein complex. The green square represents the V gene segment and the yellow triangle represents the 12 base pair recombination signal sequence. The red square represents the J gene segment and the brown triangle represents the 23 base pair recombination signal sequence. From top to bottom, the schematic shows the section of DNA to be recombined, the binding of RAG1 and RAG2 to the DNA, the single-stranded nick and formation of hairpins, and the repair and ligation of the DNA to complete the recombination. Adapted from *Kuby Immunology*, 8th ed.

Recombination is initiated when the RAG1 and RAG2 proteins form a complex and nick the DNA at the recombination signal sequences (RSS) that flank each gene segment (McBlane *et al.* 1995). These nicks cause two sealed hairpin coding ends and two blunt signal ends to form (Fugmann 2001). The hairpins are believed to be cleaved by a protein complex called Artemis which allows for DNA enzymes to fill in the complementary strands and complete the recombination process (Mansilla-Soto & Cortes 2003). Because of the numerous combinations of V, D, and J gene segments that can be recombined to form immunoglobin variable domains, this unique process in the adaptive immune system allows for a large repertoire of antigen-recognizing receptors to be created. This in turn allows for a wide range of elicited specific immune responses.

Since there is a time lag between recognition of a pathogen, proliferation of lymphocytes, development of effector cells, and action by the immune system, the adaptive immune response is more delayed than the rapid innate response. However, this initially delayed response can become faster and stronger over time with the development of immune memory. When the adaptive immune system encounters the same pathogen again, it mounts a secondary immune response with receptors that have a higher affinity for the pathogen, and in some cases, a larger immune response (Ratajczak *et al.* 2018). The adaptive immune system has B memory (B_M) cells and T memory (T_M) cells in order to create this immune memory. B_M cells have membrane bound immunoglobins with a higher affinity for the pathogen and release larger amounts of specific antibody than in the primary immune response (Ratajczak *et al.* 2018). T_M cells develop from naïve (unexposed) T-cells and are able to mount a

stronger, more effective immune response even when challenged by lower concentrations of antigen than in the initial infection (Ratajczak *et al.* 2018). The development of immune memory is unique to the adaptive immune system and allows for greater, faster immune responses with repeated exposure to pathogens.

One area of interest when studying the adaptive immune system is the TCR/CD3 complex embedded in the membrane of T-lymphocytes. The TCR/CD3 complex consists of two major protein components: TCR and CD3. Each of these major components performs different functions essential to the complex made possible by their unique physical structures. The TCR heterodimer is able to recognize a specific antigen peptide that is presented by the MHC molecule on an antigen presenting cell, and the CD3 components have immunoreceptor tyrosine-based activation motifs (ITAM) that become phosphorylated after the TCR engages with the antigen-bound MHC (Clevers *et al.* 1988). Together, this allows for the recruitment of other molecules to initiate a signaling cascade (Janeway 1992). One reason for the differing capabilities of these components is the length of the cytoplasmic tails. The TCR chains have short cytoplasmic tails and have no signaling capabilities in themselves, while the CD3 components have longer cytoplasmic tails that are capable of transducing signals across the membrane (Clevers *et al.* 1988). They must therefore work together to recognize an antigen peptide, trigger a signaling cascade, and initiate the immune response of T-cells.

The TCR/CD3 complex is of particular interest when studying the timing of onset of adaptive immunity because the genes encoding the pre-TCR/CD3 complex are some of the earliest expressed genes in the developmental pathway of the adaptive immune system. In fact, all CD3 components are expressed at the mRNA level in the

earliest identifiable thymic precursors, although surface level detection is more delayed (Wilson & MacDonald 1995). The genes encoding the pre-TCR complex, consisting of a pre-TCRα chain and a mature TCRß chain, are expressed slightly later than CD3, but before the maturation of T-lymphocytes (Wilson & MacDonald 1995).

The pre-TCR and CD3 complex is important for normal progression of early T-cell development, so the expression of CD3 and pre-TCR genes can be used as an effective marker for the onset of the adaptive immune system (Wilson & MacDonald 1995; von Boehmer & Fehling 1997). To note: the onset of the adaptive immune system refers to the earliest expression of adaptive immune genes, or earliest development of adaptive immune precursors, not the full functionality of the adaptive immune system. The TCR/CD3 complex and its precursors have been widely used to study the ontogeny of the immune system and could provide important insights on human disease and host-microbe interactions.

Human Health and the Microbiome

Animals exist with an entire ecosystem of microorganisms that live inside and on their bodies (Turnbaugh *et al.* 2007). This complex microbial ecosystem, collectively named the 'microbiome', consists of a diverse group of microbes including fungi, viruses, bacteria, archaea, protozoa and algae. The radiation of microbial eukaryotes occurred about 1.5 billion years ago, and by the end of the Cambrian explosion, all known animal phyla of microbes had appeared (Rook *et al.* 2017). Ancestral vertebrates harbored complex communities of these microbial eukaryotes and evolved an adaptive immune system because of the survival advantage gained by adding this form of

immunity to the pre-existing innate immune system (Cooper & Alder 2006; Rook *et al.* 2017).

Microbes present throughout a host can be mutualistic or pathogenic to the organism. Mutualistic microbes interact with the host by aiding in proper physiologic and metabolic processes while the microbes receive nutrition and safe residence (Ivanov & Honda 2012). Mutualistic interactions between the host and microbes can also have immunomodulatory roles (Bouskra *et al.* 2008; McFall-Ngai *et al.* 2012). Alternatively, pathogenic microbes are capable of causing disease in the host and can be detrimental to host survival. However, out of the many microbes interacting with a host, only a few cause disease and some pathogenic microbes are not pathogenic in all hosts (Casadevall & Pirofski 2000).

The majority of microbiota are contained in the gut; as many as 10 times more bacterial cells than host cells exist in the human digestive tract (Bull & Plummer 2014). Therefore, the main interface between the immune system and the microbiome occurs at the intestinal epithelium (Bull & Plummer 2014). Microbes can also be found throughout the body such as on the skin, in the mouth, in the vaginal canal, and even in the eye and placenta (Grice & Segre 2011; Neish 2014; Lloyd-Price *et al.* 2016). However, the most intraspecific microbial diversity exists in the colon (Sender *et al.* 2016). Because of these interfaces, the presence of microbiota can affect the development of the organism's immune system by continuous exposure to microbes from early development (Bull & Plummer 2014).

It is not completely understood how neonates adapt to microbial colonization during development. The first exposure of the fetus to microbes is during the passage

through the birth canal (Belkaid & Hand 2014; Lloyd-Price *et al.* 2016). Therefore, the neonate's microbiome is largely influenced by the microbes present in the mother during pregnancy and birth. There is also evidence that the mother's colostrum and breast milk are important factors for shaping the microbial environment in early life (Hunt *et al.* 2011; Belkaid & Hand 2014; Lloyd-Price *et al.* 2016). The developing immune system is skewed towards regulatory responses for recognizing self, so it is possible that this immunoregulatory environment allows for the colonization of microbes with limited inflammatory responses (Elahi *et al.* 2013; Santori 2015).

After the immune system has matured, the host can elicit an immune response to disease-causing pathogenic microbes in order to prevent infection. However, the host may also elicit an immune response to resident mutualistic microbes if they are incorrectly recognized as foreign. The immune system is trained by host-microbe interactions during development to recognize the difference between host microbes, the 'microbial self', and foreign microbes (Abraham & Medzhitov 2011). When the immune system elicits a response against the 'microbial self', a state of dysbiosis, or microbial imbalance, is created, leading to many major health problems such as Crohn's disease, ulcerative colitis, and other inflammatory diseases (Manichanh *et al.* 2006; Li *et al.* 2012; Morgan *et al.* 2012). However, it is not only interactions between the host and resident microbes that can cause disease; non-resident microbes can also interact with the host immune system and cause diseases such as atopic dermatitis, psoriasis, and Lyme disease (Gantz & Allen 2016).

Host-microbe interactions are extremely important for host health and have become an essential area of research. With the completion of the Human Microbiome

Project in 2013, our understanding of the human microbiome was greatly expanded, but there are still many questions left unanswered (Turnbaugh *et al.* 2007; The Human Microbiome Project Consortium 2012). Questions relating to host-microbe interactions are extremely relevant now during the COVID-19 pandemic. News outlets and scientists around the world are asking questions such as, how does the body interact with microbes such as viruses? How does immunity develop? Why are some people more susceptible to infection than others? Understanding generally how the human immune system interacts with a variety of microbes, including novel viruses, is essential in being able to control and treat human diseases. Additionally, understanding host-microbe interactions in the context of human disease allows us to answer questions about the importance of genetics and environment in the etiology of disease. The interactions between environment, host genetics, and microbes can determine the severity and progression of disease, yet there is still much to be understood about these interactions.

There are still many unanswered questions about host-microbe interactions, especially in the context of human disease. As ongoing research continues to pursue answers to these questions, manipulative studies may be necessary in order to understand the molecules, processes, and specific interactions involved. By utilizing animal models in studies of host-microbe interactions, these manipulative studies can be more easily performed.

Animal Modeling

Animal models are widely used in biological and biomedical research as organisms with similarities to humans. Animals can reflect the processes and

physiology of humans, so they provide a different avenue for studying human disease

and immune processes in studies from basic science research to vaccine and treatment

development (Barré-Sinoussi & Montagutelli 2015). Specifically, immunological

studies rely on animal models with immune systems reflective of humans in order to

understand host-microbe systems and the genetics of innate and adaptive immunity

(Chandler *et al.* 2011; Milligan-Myhre *et al.* 2011; Gootenburg & Turnbaugh 2011;

Milligan-Myhre *et al.* 2016). Current models, such as mice and zebrafish, can be

extremely effective because they mimic the human immune system through conserved

pathological conditions (Rivera & Tessarollo 2008). However, although physiologically

similar, these isogenic lines of animal models are challenged by a lack of genetic

diversity, as found in human populations, and for mice, by the invasive procedures

required to work on mammalian models (Rivera & Tessarollo 2008). There is a need for

a genetically diverse, non-mammalian model in order to study the immune system and

host-microbe interactions in the context of genetic variation. This gap is being filled by

fish as animal models including threespine stickleback, zebrafish, and other fish species

(Davis 2004; Zhu *et al.* 2013).

Fish Immunology

Fish are becoming more popular models for immunological studies, but there are

foundational questions that must be answered to effectively utilize these models, such

as, when do the cells and molecules of the adaptive immune system first begin to

develop? Previous studies on the development, or onset, of adaptive immunity in

mandarin fish (*Siniperca chuatsi*) and multiple teleost fishes have shown that externally

fertilized fish do not require the adaptive immune system for survival until they have

hatched (Tian *et al.* 2009; Uribe *et al.* 2011). Therefore, the adaptive immune system is likely onset when there is first exposure to external pathogens after the embryo is exposed to the environment, such as after hatching. However, the methods used to determine onset are variable, and different species develop at different rates, so the reported onset of adaptive immunity in fish species ranges from 11 hours post fertilization in zebrafish (*Danio rerio)* to 26 days post hatching in mandarin fish (*Siniperca chuatsi*) (Willett *et al.* 1999; Tian *et al.* 2009; Uribe *et al.* 2011). Interestingly, the onset of adaptive immunity in two teleost species, marine medaka (*Oryzias melastigma*) and zebrafish (*Danio rerio*), correlates with a common developmental time point: the opening of the mouth and/or anus (Kimmel *et al.* 1995; Swarup 1958; Iwamatsu 2004). The onset of the adaptive immune system could also be influenced by how long larvae are sustained on yolk, possibly because of maternally transferred immune factors present in the yolk (Zhang *et al.* 2013).

In addition to microbial exposure, there are many environmental factors that could cause variation in the development of adaptive immunity in fish including natural environmental factors such as temperature, salinity, or season, or artificial environmental factors such as pollutants or human confinement (Bly *et al.* 1997; Le Morvan *et al.* 1998; Cheng & Chen 2000; Baze *et al.* 2011; Birrer *et al.* 2012; Petersen *et al.* 2015; Gobler & Baumann 2016; Cabillon & Lazado 2019). The habitat-specific microbiome can also influence the fish immune system (Hooper *et al.* 2012). Due to the strong influence of environment on the development of adaptive immunity, it is important to utilize fish models that come from various well-understood environments in order to understand the influence of environmental factors across populations.

Additionally, it is important to include wild-caught fish in immunology studies because wild populations have been reared in environments with different characteristics and microbes. In order to accurately represent the human immune system in the context of genetic and environmental variation, fish models should also represent a range of populations from different environments. There are differences in the expression of functional immune parameters and immune genes between wild and lab-bred animal models, so it is important to sample across populations to have a better understanding of immune function and development (Huang *et al.* 2016; Abolins *et al.* 2017). One species of fish that allows for easy sampling across a range of populations is the threespine stickleback fish.

The Threespine Stickleback Model Organism

Threespine stickleback (*Gasterosteus aculeatus*), a teleost fish, exists in various oceanic, freshwater, and brackish environments throughout the Northern Hemisphere ranging from the open ocean to small floodplain potholes (Bell & Foster 1994). Some freshwater populations are fairly young because the retreat of Pleistocene glaciers around 12,000 years ago forced marine threespine sticklebacks to colonize and adapt to newly made freshwater environments (McKinnon & Rundle 2002; Jones *et al.* 2012). Additionally, some young Alaskan freshwater populations were formed from previously marine environments that became freshwater ponds by the 1964 Great Alaska Earthquake in Prince William Sound and the Gulf of Alaska (Lescak *et al.* 2015). These new populations of threespine stickleback can undergo rapid differentiation when they become isolated, but do not go as far as to become distinct species (Bell & Foster 1994; McKinnon & Rundle 2002). In addition to phenotypic divergence between populations,

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threespine stickleback exhibit high genetic diversity between populations and within

families (Bell & Foster 1994; McKinnon & Rundle 2002; Cresko *et al.* 2004).

Figure 4: An adult male threespine stickleback.

The threespine stickleback is externally fertilized and can vary in time to

hatching depending on the incubation temperature and population of fish. Within the

first day post fertilization, the blastodermic cap, a mass of cytoplasm at one pole of the

egg, is formed and is segmented many times (Swarup 1958). After 1 day post

fertilization, there is a clear germ ring seen around the edge of the blastoderm; at this

stage, the blastoderm almost appears to be a mushroom cap over the subgerminal

cavity. By 2 days post fertilization, there is a well-established embryonic axis and the

development of the central nervous system causes the embryo to thicken, making it

obvious to the naked eye (Swarup 1958). The optic, cardiac, and central nervous

systems continue to develop and by about 5 days post fertilization, the eyes are

prominent and pigmented, the heart is beating vigorously, the tail shows occasional

muscular movements, and melanophores begin to appear along the dorsal side of the

body (Swarup 1958). At 6 to 7 days post fertilization, by lashing its tail around and

vibrating the pectoral fins, the head is pushed against the chorion – the outer membrane

surrounding the embryo – and the chorion ruptures allowing the larval fish to free itself

from the egg (Swarup 1958). At or shortly after hatching, the hindgut is visible and opens by the anus, and the first lymphoid cells may be present in developing lymphoid organs: the thymus, spleen, and head kidney (analogous to the human adrenal gland and the major hematopoietic organ in fish) (Swarup 1958; Zapata *et al.* 2006; Geven & Klaren 2017). Following hatching, the larval fish absorbs its yolk and continues to develop with a straightening of the head, a completely developed jaw and functional mouth, an elongated intestine, and a visible swim bladder (Swarup 1958). Additionally, the TCR genes of the TCR/CD3 complex may be expressed by around 2 days post hatching as in other teleost species (Zapata *et al.* 2006). By around 4 days post hatching, the yolk has been completely absorbed and the fish must be provided food and moved into a larger water system in the laboratory.

Threespine stickleback are excellent biological models because they are widely distributed throughout the Northern hemisphere, can be easily caught and maintained in the laboratory, and because there are such striking differences between even adjacent habitats (Bell & Foster 1994). The evolutionary history and development of threespine stickleback are well understood, which also contributes to their use as a model (Swarup 1958; Bell & Foster 1994; McKinnon & Rundle 2002). As animal models, the threespine stickleback has been used to answer many questions including those about the genetic basis for the repeated evolution of armor loss, the differences in immune responses to microbiota between populations, intestinal inflammation, and population genetics (Cresko *et al.* 2004; Milligan-Myhre *et al.* 2016; Small *et al.* 2017; Small *et al.* 2019; Beck *et al.* 2020). While threespine stickleback fish have been used as an

effective model for a variety of studies, the species is still emerging as a model for studies of the immune system.

Threespine stickleback fish can be used as an outbred immunogenetics model appropriate for studies of the immune system within the context of genetic variation. These teleost fish are an example of outbred 'evolutionary mutant models', defined by Albertson *et al.* 2009 as an assemblage of related organisms in which certain populations express a phenotype that mimics human disease and exhibit genetic variation from natural selection and genetic drift. The natural genetic variation and vertebrate immune systems of threespine stickleback allow the species to be developed as an animal model that can mimic human disease phenotypes (Albertson *et al.* 2009; Milligan-Myhre *et al.* 2016). Additionally, threespine stickleback are genetically tractable organisms with a well-annotated genome that is helpful for immunogenetics studies. Similar to zebrafish, threespine stickleback are easily reared in the laboratory through external fertilization with high fecundity allowing for the facilitation of developmental assays and the study of immune development. For these reasons, the threespine stickleback fish is presented as a novel model for immunogenetics studies to be used for controlled manipulative host-microbe interaction studies.

Chapter 2

Introduction

Vertebrates possess an adaptive immune system that is able to initiate responses against a variety of pathogens such as viruses, fungi, and bacteria (Delves & Roitt 2000). These interactions between the immune system and the microbial environment, or host-microbe interactions, are important for maintaining homeostasis with mutualistic microbes and maintaining a healthy host (Delves & Roitt 2000; Hooper et al. 2012). When the relationship between the host and microbes goes awry, major health problems can occur including Crohn's disease, ulcerative colitis, Lyme disease and atopic dermatitis (Manichanh et al. 2006; Li et al. 2012; Morgan et al. 2012; Gantz & Allen 2016). Host-microbe interactions in the context of human disease have become a popular area of study, but our current understanding of these interactions and chronic immune disorders is challenged by not knowing when the onset of the adaptive immune system occurs. Understanding the onset of adaptive immunity is important for advancing our knowledge of host-microbe interactions and disease etiologies by determining when hosts first develop the cells and molecules needed to respond to pathogens and microbes.

The adaptive immune system is a large, complex system that has evolved in vertebrates and been maintained for the survival advantage it provides compared to possessing the pre-existing innate immune system alone (Cooper & Alder 2006; Hirano *et al.* 2011; Zhao & Elson 2018). This branch of the immune system is advantageous because it allows for specific immune responses and the development of immune

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memory – secondary responses with a higher affinity and greater response to the pathogen (Ratajcazk *et al.* 2018). Previous studies have determined the molecules, mechanisms, and processes associated with adaptive immunity, but the onset of the adaptive immune system has been conclusively reported in very few species.

In studying the onset of adaptive immunity, one area of interest is the TCR/CD3 complex that is important for the activation of T-lymphocytes. The TCR/CD3 complex consists of a heterodimer (TCRα and TCRß) and invariant CD3 components (CD3δ, CD3γ and two CD3ε) that are together able to recognize a specific antigen peptide, transduce the signal across the membrane, and trigger a signaling cascade to activate the T-cell (Clevers *et al.* 1988; Janeway 1992). The TCR/CD3 complex is of particular interest when studying the onset of adaptive immunity because it is important for early T-cell development. Additionally, the genes encoding the TCR/CD3 subunits are some of the earliest expressed adaptive immune genes (Wilson & MacDonald 1995). Therefore, the expression of CD3 and TCR genes can be used as effective markers for the onset of the adaptive immune system (Wilson & MacDonald 1995; von Boehmer & Fehling 1997).

Many ongoing studies on the onset of adaptive immunity utilize isogenic lines of animal disease models, i.e. mice and zebrafish, and do not include the use of natural models from different environments. It is important to utilize immunological models that can effectively mimic human diseases within the context of genetic variation and different environmental histories because the host's genetic background and environmental exposure are extremely influential on susceptibility to disease and the progression of disease states (Zhernakova *et al.* 2008; Rivera & Tessarollo 2008).

Additionally, different environmental histories are often correlated with differences in immune activity, so it is vital to include natural models in immunological studies (Le Morvan *et al.* 1997; Cheng & Chen 2000; Matthews *et al.* 2010). We address the challenges of current animal models by presenting threespine stickleback as an emerging model in immunogenetics.

Threespine stickleback fish (*Gasterosteus aculeatus*) can be used as a genetically diverse outbred model appropriate for immunological studies. The teleost fish exists in various oceanic, freshwater, and brackish environments through the Northern Hemisphere and exhibits genetic diversity among populations and within families (Bell & Foster 1994; McKinnon & Rundle 2002; Cresko *et al.* 2004). Additionally, these small fish are genetically tractable organisms with a well-annotated genome allowing for immunogenetics studies. Similar to zebrafish, threespine stickleback are easily reared in the laboratory through external fertilization and produce many offspring allowing for the facilitation of developmental assays and the study of immune development. The natural genetic variation and vertebrate immune system of threespine stickleback allows for the species to developed as an effective immunological model (Albertson *et al.* 2009; Milligan-Myhre *et al.* 2016). This non-mammalian model has already been previously used to study immune responses in various environments, but there are fundamental questions left unanswered that are necessary for progressing threespine stickleback as a natural disease model (Kurtz *et al.* 2006; Bolnick *et al.* 2015; Milligan-Myhre *et al.* 2016; Small *et al.* 2017; Small *et al.* 2019; Beck *et al.* 2020).

It is currently unknown when adaptive immunity is onset in threespine stickleback, and if the adaptive immune system is uniformly onset between populations. In this study, we attempt to answer these questions and chose to focus on the onset of *cd3d*, a gene involved with the CD3 delta subunit of the TCR/CD3 complex, in two populations of threespine stickleback. Similar work determining the onset of adaptive immunity in other fish has produced a wide range of results, from 11 hours post fertilization in zebrafish (*Danio rerio*) to 26 days post hatching in mandarin fish (*Siniperca chuatsi*) (Willett *et al.* 1999; Tian *et al.* 2009). We determined a timepoint in which *cd3d* is first expressed in threespine stickleback, with population level variation indicating that some individuals may exhibit expression at an earlier developmental time point. These findings have broader implications for presenting threespine stickleback as an emerging immunogenetics model and for understanding the onset of adaptive immunity and host-microbe interactions.

Methods

Populations, Husbandry and Crosses

      I generated families of threespine stickleback from two Alaskan populations, an anadromous population – born in freshwater, but live in saltwater and return to freshwater to spawn – Rabbit Slough (N 61.5595, W 149.2583), and a freshwater population, Boot Lake (N 61.7167, W 149.1167) (Figure 5). These populations have been maintained in the laboratory for at least ten generations. Three families (the offspring of one male and one female) were generated from Rabbit Slough and four families were generated from Boot Lake using *in vitro* crossing outlined by Cresko *et al.* 2004. Each of the Rabbit Slough parents used came from a distinct family, while at least one parent in each of the Boot Lake crosses came from a single family. The developing embryos were raised in stickleback embryo media in an incubator at 20 ºC until 9 days post fertilization (dpf) and then moved to the recirculating water system in the Cresko Lab fish facility. Water temperature was kept at 20 °C with a salinity of 2-4 parts per thousand (PPT). The fish were housed in "summer" light conditions of 16 hours of daylight and 8 hours of night and were fed daily with hatched brine shrimp naupli and fry food (Ziegler AP100 larval food).
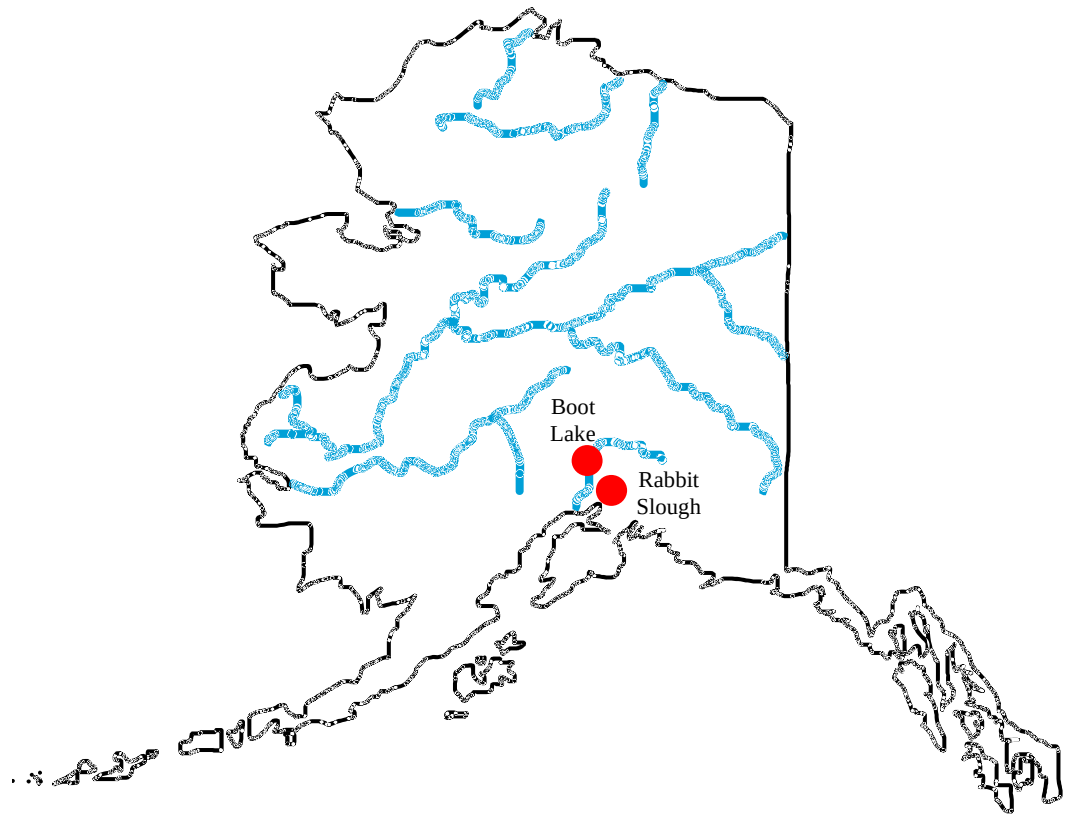
Figure 5: Map of major rivers in the state of Alaska. A map of Alaska, USA showing the major rivers in blue. The collection sites for two lab-adapted threespine stickleback lines, Boot Lake and Rabbit Slough, are indicated with red dots along the Susitna River.

Developmental Time Series

To analyze the expression of *cd3d*, we generated developmental time series of larval fish from 0 to 21 days post hatching (dph) from each family in each population. Three to six fish were grouped and preserved at each time point from each family, depending on the number of embryos available in a clutch. Due to limitations of embryos, not all series extended until 21 dph (Table 1). Fish were euthanized with MESAB according to IACUC approved methods and preserved in RNALater to preserve the integrity of RNA for extraction. Preserved individuals were kept at 4 °C for 24 hours then stored long-term at -20 °C.

Table 1: Lab-adapted individuals preserved for the creation of a developmental time series

Population	Individuals preserved	Time Range
Rabbit Slough	44	0 dph to 14 dph
Rabbit Slough	95	0 dph to 21 dph (except 20 dph)
Rabbit Slough	52	0 dph to 21 dph (except 11 dph, 15 dph and 16 dph)
Boot Lake	51	0 dph to 21 dph (except 13 dph, 18 dph and 19 dph)
Boot Lake	36	0 dph to 21 dph (except 16 dph, 18 dph and 20 dph)
Boot Lake	34	0 dph to 10 dph, and 14 dph
Boot Lake	100	1 dph to 22 dph

After all developmental time series were created, we isolated the heads of preserved fish by cutting directly behind the pectoral fin. By isolating the head at the pectoral fin, the major lymphoid organs that express adaptive immune genes such as the thymus and head kidney will be included in the caudal section used for assays. The heads were preserved in 100 μL of RNALater and kept at 4 °C to stabilize the RNA in the sample and the remaining portions were frozen at -20 °C to prevent degradation of DNA.

Identification of Immune Marker Genes

We searched the threespine stickleback reference genome on Ensembl for annotated early adaptive immune genes associated with the TCR/CD3 complex. The only annotated TCR/CD3 gene in the threespine stickleback genome is *cd3d*, the gene encoding the delta subunit of the CD3 complex. A BLAST search compared the

sequence of *cd3d* (exon two) against the threespine stickleback genome and revealed no paralogues.

RNA Isolation, First Strand Synthesis and PCR

From the developmental time series generated for seven lab-adapted families, RNA was isolated from the heads of three individuals from each population at selected time points using the Direct-zol RNA MiniPrep Kit (R2052; Zymo Research, Irvine, CA, USA) and quantified using the Qubit RNA High Sensitivity Assay Kit (Q32852; Invitrogen). At each time point (except 10 dph) three individuals from one family in each population were selected for RNA isolation (Table 2). At 10 dph, one individual from three different families in each population were selected for RNA isolation. Then, the isolated RNA was converted to single-stranded cDNA by first strand synthesis performed using standard protocols.

Time points from the developmental time series were selected using a binary classification decision tree that allowed for a methodical approach to narrowing the time range of 0 to 21 days post hatching (Figure 6). By assaying fish at a predetermined midway point, we were able to work through each layer of the tree, choosing the next time point to assay based on the results of the previous step. Each step through the decision tree allowed for the possible time frame to be cut in half, e.g. confirmed expression of *cd3d* at 10 dph eliminates the need to assay any fish between 11 dph and 21 dph.
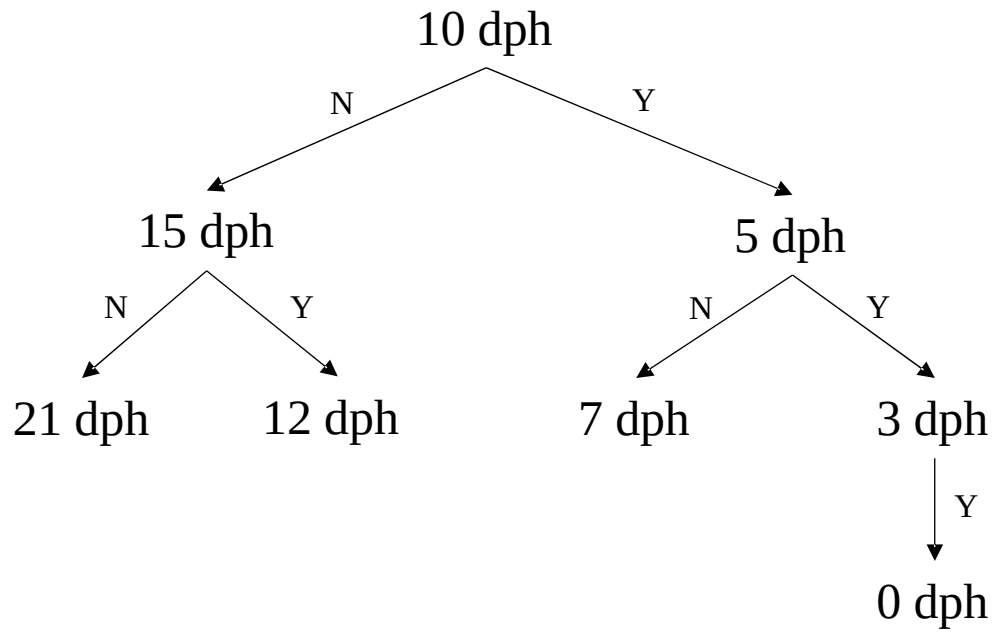
Figure 6: Binary classification decision tree. "Y" indicates yes, there was expression of the gene in the previous step. "N" indicates no, there was not expression of the gene in the previous step. The arrows were followed to determine the next timepoint to be assayed.

Table 2: Lab-adapted individuals assayed for analyzing the expression of the *cd3d* gene

Population	Number of Fish	Time Point
Boot Lake	1	10 dph
Boot Lake	1	10 dph
Boot Lake	1	10 dph
Rabbit Slough	1	10 dph
Rabbit Slough	1	10 dph
Rabbit Slough	1	10 dph
Boot Lake	3	5 dph
Rabbit Slough	3	5 dph
Boot Lake	3	3 dph
Rabbit Slough	3	3 dph
Boot Lake	3	0 dph
Rabbit Slough	3	0 dph

The synthesized *cd3d* cDNA was used in polymerase chain reactions (PCR) to qualitatively determine if the target adaptive immune gene is expressed at the selected timepoints of the developmental time series. Forward and reverse primers for the adaptive immune gene, *cd3d*, were designed with Geneious Prime 2019.1.1 (https://www.geneious.com) using the threespine stickleback reference genome from Ensembl (Kearse *et al.* 2012) (Table 3). In order to confirm the effectiveness of PCR, we used a housekeeping gene as a positive control in parallel to the adaptive immune gene. Primers for *rpl13a*, a threespine stickleback housekeeping gene, were also designed with Geneious Prime 2019.1.1 based on primer sequences reported by

Hibbeler *et al.* 2008. All primers were designed across exons to enable joint use as a
positive control for genomic DNA amplification.

PCR reactions used the parameters outlined in Table 3. Following PCR, we used
gel electrophoresis to visualize the presence or absence of cDNA for *cd3d* and *rpl13a*.
Individuals were classified as having expression of the early adaptive immune gene or
having no expression of the gene based on the presence or absence of a band from the
cd3d PCR product (Figure 7).

Table 3: PCR parameters for *cd3d* and *rpl13a*

Gene Name	*cd3d*	*rpl13a*
Forward Sequence	GCTGTGGTTCCTGTCCTA	TATCCCTCCGCCCTACG
Reverse Sequence	GGTAGTGATCGTCGGTGG	GCAACCTTGGTCAACTTGAACA
Annealing Temperature	54°C	54°C
Number of Cycles	48	48
Primer Volume (per 20 μL reaction)	1.5 μL	0.6 μL
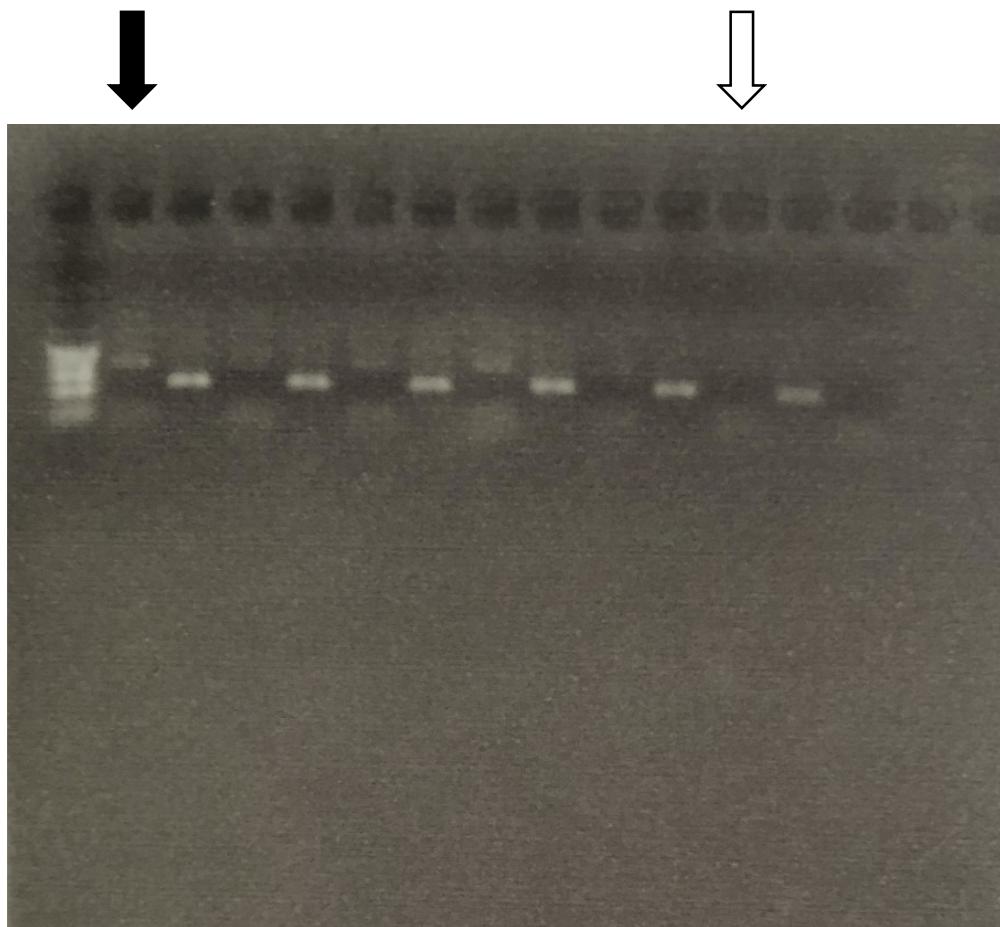
Figure 7: Example of positive expression of *cd3d* on agarose gel. PCR product from six 3 dph individuals run on a 2% agarose gel. Lane 1 is a 1 kb plus ladder. Lanes 2, 4, 6, 8, 10, and 12 are each the product of cDNA from a different individual with *cd3d* primer. Lanes 3, 5, 7, 9, 11, and 13 are each the product of cDNA from a different individual with rpl13a primer. Lanes 2-7 are Boot Lake individuals and lanes 8-13 are Rabbit Slough individuals. The black arrow represents a lane classified as "expression present". The white arrow represents a lane classified as "no expression".

Results

cd3d *Is Expressed in Threespine Stickleback by 10 Days Post Hatching*

To determine the developmental timing of *cd3d* onset, we assayed larval fish for gene expression from 0 dph to 10 dph in two lab-adapted populations of threespine stickleback, Boot Lake and Rabbit Slough. Of the three Boot Lake individuals assayed at each of four early time points (0 dph, 3 dph, 5 dph, and 10 dph), at least one individual showed expression of *cd3d* at each time point (Figure 8a). Two of the three fish assayed at 0 dph had expression of *cd3d*, but these results could not be replicated and therefore are not conclusive. At 3 dph, all assayed individuals showed expression of the adaptive immune gene. This pattern was also observed at 5 dph and 10 dph. Therefore, we propose the onset of adaptive immunity to be prior to 3 dph in the Boot Lake population (Figure 8b).

The individuals from Rabbit Slough families showed expression in at least one individual at 3 dph, 5 dph, and 10 dph (Figure 8a). There was no expression of *cd3d* at 0 dph, but two of the three individuals assayed at 3 dph and 5 dph had expression. In comparison to Boot Lake, it was not until 10 dph that all three Rabbit Slough individuals showed expression of *cd3d.* Therefore, we propose the onset of adaptive immunity to be prior to 10 dph in the Rabbit Slough population (Figure 8b). Considering both populations, by 10 dph, all assayed individuals showed expression of the *cd3d* gene (Figure 8a).
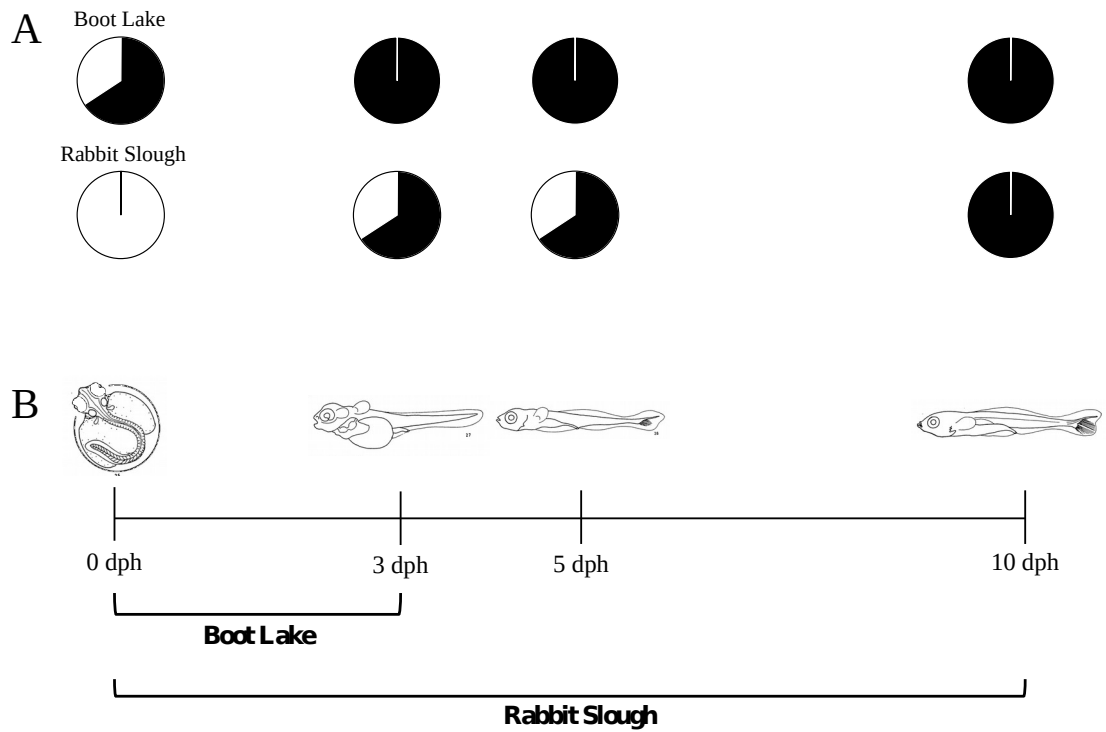
Figure 8: *cd3d* expression patterns. (a) The expression of *cd3d* in 3 individuals at each developmental time point (0 dph, 3 dph, 5 dph, and 10 dph) from each population. Each pie chart is above the corresponding timepoint listed in panel B. The pie chart represents the proportion of individuals that had expression of *cd3d*. The black represents on and the white represents off. (b) The proposed range of onset for the two assayed populations of threespine stickleback. Range is based on time points from no expression of *cd3d* in at least one individual to earliest expression of *cd3d* in all three individuals. The developmental stages of threespine stickleback are overlaid and correlate to the pie charts above. Stickleback development drawings adapted from Swarup 1958.

Discussion

We have determined a window for the onset of adaptive immunity that was earlier than previously thought by analyzing the expression of *cd3d* in threespine stickleback. Manipulative studies of the adaptive immune system could now be performed within this narrower timeframe. Understanding these patterns could also be useful for advancing threespine stickleback as an emerging model for immunological studies in the context of genetic and environmental diversity.

Hypotheses

We expected to find the onset of the adaptive immune system at or near hatching in threespine stickleback because of the timing of microbial exposure during development. Threespine stickleback are fertilized and develop within the chorion, the outer membrane surrounding the embryo, for about 6 to 7 days after fertilization in our lab populations. Then, the larval fish hatch out of the chorion and are exposed to the environment at 0 days post hatching, remain in embryo media in an incubator until 9 to 10 days post fertilization, and then are housed in the fish facility's recirculating water system. Each of these developmental stages can expose the fish to different microbes from the environment. Over these first days of development, the onset of the adaptive immune system would likely occur to face the challenge of microbes in the environment. If the fish are not challenged by microbes prior to hatching, assuming a sterile interior to the chorion, then we would expect the adaptive immune system to onset at a developmental time point after hatching.

Other teleost fish species such as marine medaka (*Oryzias melastigma*) and zebrafish (*Danio rerio)* express TCR/CD3-associated genes including *tcrß* and *tcrα* at 5

days post hatching and 4 days post fertilization, respectively (Lam *et al.* 2003; Danilova *et al.* 2004; Zapata *et al.* 2006; Seemann *et al.* 2017). In our own study using a teleost model, we found that threespine stickleback express *cd3d* as early as 0 dph to 3 dph in some individuals. Such variation in the timepoint of onset is expected between species, as these three teleost fishes develop at different rates. However, the expression of TCR/ CD3-associated genes are consistently expressed at a similar developmental time point. In each of the three teleost species, the earliest expression of TCR/CD3 related genes corresponds with the opening of the mouth and/or anus (Swarup 1958; Kimmel *et al.* 1995; Iwamatsu 2004). These openings are entryways for microbes from the environment to enter the fish and colonize. There could be a correlation between the opening of the mouth/anus and the onset of the adaptive immune system that could then be extrapolated to other fish species. These expression patterns are not true of all adaptive immune markers, but of the TCR/CD3 complex, specifically.

cd3d *Expression Indicates the Onset of the Adaptive Immune System by 10 Days Post Hatching*

Of the three Boot Lake individuals assayed at each of four early timepoints (0 dph, 3 dph, 5 dph, and 10 dph) at least one individual showed expression of *cd3d* at each time point while in Rabbit Slough there was at least one individual showing expression only at 3 dph, 5 dph, and 10 dph. These earliest expression patterns are in line with the hypothesis that the adaptive immune system would be initially onset between 0 dph and 3 dph. If there is expression seen at a given timepoint, we determine that the onset of the *cd3d* gene is at or prior to that time point. Even with variable

expression patterns between families, there seems to be expression of *cd3d* in some individuals beginning around 3 dph in each population as a whole.

Interestingly, there was expression of *cd3d* in two individuals from the Boot Lake population at 0 dph, but no expression from Rabbit Slough individuals at the same time point. So, considering the species overall, *cd3d* may be expressed at 0 dph or earlier in some threespine stickleback. However, when the individuals in both populations were re-assayed for confirmation of these results at 0 dph, there was no expression in any individuals from either population. Therefore, the results of gene expression at 0 dph were inconclusive. It is more likely that the onset of the adaptive immune system occurs after 0 dph because other fish species, including some teleost fish, are known for having a delayed onset of adaptive immunity from hatching (Chantanachookin *et al.* 1991; Willett *et al.* 1997; Magnadottir *et al.* 2005; Seemann *et al.* 2017). To conclusively determine the earliest onset of the adaptive immune system, a larger selection of individuals from each family and population would need to be assayed. While variation is expected within and between families and populations, a general conclusion about whether or not *cd3d* is onset at 0 dph could be made through future studies.

By determining the onset of *cd3d* expression, we attempted to pinpoint the first time point at which the adaptive immune system begins to develop, or the onset of the adaptive immune system. We chose to use *cd3d* to study the onset of adaptive immunity because T-lymphocytes are unable to recognize antigen peptides without surface expression of the protein complex, and therefore, the adaptive immune system could not be fully functional without proper expression of the TCR/CD3 complex (Weiss & Stobo

1984). Because of its essential role in developing thymocytes, *cd3d* can serve as a marker of some of the earliest gene expression in the adaptive immunity developmental pathway (Clevers et al. 1988). Using this adaptive immune marker, we found that by 10 dph, threespine stickleback individuals express the *cd3d* gene, indicating that the onset of the adaptive immune system occurs by that time point. This proposed timing for the onset of adaptive immunity is much earlier than previously thought, as studies doing similar work have analyzed gene expression in fish at upwards of 28 dph so future studies will be able to perform analyses and manipulations at much earlier time points (Hasse *et al.* 2016; Scharsack *et al.* 2017).

Expression of cd3d *May Also Indicate the Onset of the Innate Immune System*

    cd3d, while a useful marker for the adaptive immune system and the delta component of the TCR/CD3 complex, may also indicate the onset of the innate immune system (Lanier *et al.* 1992). The delta subunit of CD3 can also be expressed in developing natural killer (NK) cells of the innate immune system (Lanier *et al.* 1992). Therefore, while the CD3δ subunit is expressed on the surface of T-cells early in development, it is also possible that early innate immune cells will have a detectable CD3δ subunit (Lanier *et al.* 1992). In that case, expression of the *cd3d* gene may indicate the onset of the adaptive or innate immune system.

    To confirm the onset of the adaptive immune system versus the innate immune system, the expression of other TCR associated genes, e.g. *rag1, rag2, tcrα* and *tcrβ,* should be analyzed in parallel with CD3 genes. The CD3 delta subunit, encoded by *cd3d,* is one of the earliest expressed chains in the TCR/CD3 complex and is essential for proper surface expression and function of the T-cell receptor (Clevers et al. 1988;

von Boehmer & Fehling 1997; Araki et al. 2005). Without the co-expression of CD3 with the T-cell receptor, the T-cell does not transport the TCR/CD3 complex to the surface (Weiss & Stobo 1984; Clevers et al. 1988; Alarcon et al. 1988).Therefore, T-lymphocytes are not fully functional without the co-expression of CD3 and TCR, so by analyzing the expression of these genes in parallel, the onset of adaptive immunity could be more confidently reported (Clevers *et al.* 1988; Araki *et al.* 2005). However, TCR genes such as *tcrα* and *tcrβ* are not yet annotated in the threespine stickleback genome.

Variation in cd3d *Expression Patterns May Indicate Population Level Variation*

An assay of threespine stickleback individuals in the Boot Lake and Rabbit Slough populations revealed intraspecific variation in the expression of *cd3d* which may indicate population level variation. Boot Lake individuals showed expression of the *cd3d* gene in all individuals starting at 3 dph and there was variable expression between the three individuals at 0 dph, however the results at this earliest time point were unreliable (Figure 8a). In comparison, Rabbit Slough individuals showed expression of the gene in all individuals at 10 dph but showed variable expression between individuals at 5 dph and 3 dph, and no expression at 0 dph (Figure 8a). This may indicate a later onset of *cd3d* in the Rabbit Slough population and that Boot Lake has a unique, earlier expression of *cd3d*.

The possibility of *cd3d* onset occurring later in the anadromous Rabbit Slough population is a credible possibility because fish from marine environments may have delayed ontogeny of adaptive immune cells (Magnadottir *et al.* 2005). It is possible that threespine stickleback populations follow this same pattern of variation in the onset of

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adaptive immunity across environmentally distinct populations. In order to fully answer the question of population level variation in the onset of adaptive immunity, it is necessary to use a greater sample size from each population and to assay across many different populations of threespine stickleback from different environments. While this study serves to suggest the potential of population level variation, more work is needed in order to accurately and quantitatively determine difference in the timing of onset of adaptive immunity between populations of threespine stickleback.

Conclusions

By analyzing the expression of an early adaptive immune marker gene across different developmental stages of threespine stickleback, we were able to indicate that the onset of the adaptive immune system occurs at or before 10 dph in this species. This study begins to answer questions about the onset of adaptive immunity and population level variation in order to progress threespine stickleback fish as a novel, non-mammalian natural model appropriate for immunogenetics studies. Our findings, although in a teleost model, may be representative of the vertebrate immune system in general and have potential for being reflective of the human immune system. With genetically diverse models such as threespine stickleback, we will continue to further our understanding of host-microbe interactions and the immune system through animal models and continue to progress the field of immunology as it attempts to better understand human health and disease.

Chapter 3: Future Directions

Within the field of immunology, there is a need for non-mammalian, outbred evolutionary mutant models in order to accurately mimic human immune function and disease states in the context of genetic variation. Our current understanding of many diseases is in part challenged by the invasive nature of prenatal tests needed to study development in mammalian models, and by the complexity of genetic variation that plays a large role in etiologies of immune disease. Therefore, the development of an outbred, non-mammalian model is essential for progressing our understanding of immunogenetics and the immune system. Threespine stickleback are presented as an emerging immunogenetics model due to their natural genetic variation and diversity of environmental histories that allow for further studies into the onset of the immune system and how genetic variation impacts phenotypic variation of immune diseases. The use of this novel model creates a wide array of opportunities for further studies in immunogenetics.

One important study related to the onset of the adaptive immune system in threespine stickleback would be to explore the onset of other early adaptive immune markers such as genes associated with the TCR heterodimer of the pre-TCR/CD3 complex, *tcrα* and *tcrβ*, and genes associated with the V(D)J recombination of immunoglobins, *rag1* and *rag2*. The TCR genes encoding for the alpha and beta subunits of the heterodimer form the core of the TCR/CD3 complex and make up some of the earliest constructed units of the pre-TCR/CD3 complex (Wilson & MacDonald 1995). Therefore, the expression of these genes may indicate an even earlier process in the development of the adaptive immune system than the expression of CD3 genes.

Additionally, the RAG genes, essential for the recombination of immunoglobins, must be expressed in order for the adaptive immune system to function properly. When mutations occur in *rag1* and *rag2*, immunodeficiencies such as severe combined immunodeficiency (SCID) can occur due to a complete absence of functional T- and B-cells in the individual (Corneo *et al.* 2001; Cossu 2010). The expression of *rag1* and *rag2* is therefore essential for normal functionality of the adaptive immune system, so the RAG genes could be used as effective markers of the onset of the adaptive immune system. These markers could be used widely across many species of animal models with annotated orthologues, such as some species of fish, rodents, and primates to study the onset of adaptive immunity.

In addition to utilizing a broader range of adaptive immune markers, studies on the onset of adaptive immunity would benefit from a quantitative analysis of TCR/CD3 gene and RAG gene expression. This study was able to qualitatively analyze the expression of *cd3d* with standard PCR and gel electrophoresis, but quantitative polymerase chain reaction (qPCR) would provide a quantitative assessment of gene expression by monitoring the amplification of a specific cDNA. A quantitative measure of expression could provide a more specific and trustworthy result for the onset of adaptive immunity by removing any limitations set by trying to visualize small concentrations of amplified cDNA at early timepoints. qPCR would also allow for a more detailed report of expression patterns by immune marker genes throughout early development.

Additionally, we can easily develop laboratory environmental manipulation assays for threespine stickleback fish. There are many environmental factors that could

cause changes in the development of adaptive immunity including water conditions such as salinity, temperature, or exposure to compounds such as lithium chloride (LiCl) (Le Morvan *et al.* 1998; Cheng & Chen 2000; Matthews *et al.* 2010; Baze *et al.* 2011; Birrer *et al.* 2012; Petersen *et al.* 2015). These various environmental differences have a strong effect on the development of the immune system and can be exploited to develop manipulative studies in the emerging threespine stickleback model (Gobler & Baumann 2016; Cabillon & Lazado 2019).

In future studies, it will be important to take advantage of the environmental diversity of threespine stickleback populations and include wild-caught fish in immune development studies. Between wild-caught and lab-bred populations, and even between different wild-caught populations, there may be variations in the microbial environment that would present different challenges to the fish. Such variations and their effects on the adaptive immune system have been studied in mammalian models and have revealed differences between the adaptive immune systems of wild-caught and lab-bred animals (Abolins *et al.* 2017) Therefore, similar patterns may be observed in non-mammalian models and there may be differences in the onset of the adaptive immune system between lab-adapted and wild-caught populations of threespine stickleback. By utilizing a wider selection of populations from different environments, studies can also analyze the effects of genetic similarity versus environmental similarity on the onset of the adaptive immune system.

To prepare for further studies on population level variation and studies utilizing populations from different environments, we generated families of threespine stickleback from two Oregon populations, the freshwater Green Island population

(44°8'42.02"N 123°7'4.88"W), and the anadromous Cushman Slough population

(43°59'224"N 124°2'42.94"W) (Figure 9). Adult fish were caught in the summer of

2019 by other members of the Cresko Lab and crosses were made in the field. The

embryos were brought back to the Cresko Lab fish facility and bleached to remove

pathogens at 48-60 hours post fertilization by soaking in a working stock solution of

bleach (500 μL/1L embryo medium) for 1.5 minutes. Then, the embryos were rinsed

three times in fresh embryo medium and reared in normal conditions. Fish were raised

according to the same conditions used for lab-adapted families (Cresko *et al.* 2004).

Cushman Slough individuals were grouped on the day they hatched regardless of family

while Green Island individuals were grouped on the day they hatched according to

family. From these fish, we created developmental time series from 0 to 21 dph in each

family from each population, but with many incomplete series due to embryo

limitations (Table 4). The Green Island and Cushman Slough developmental time series

fish are currently preserved in RNALater and kept at -20 °C for long term storage to be

utilized in future studies.
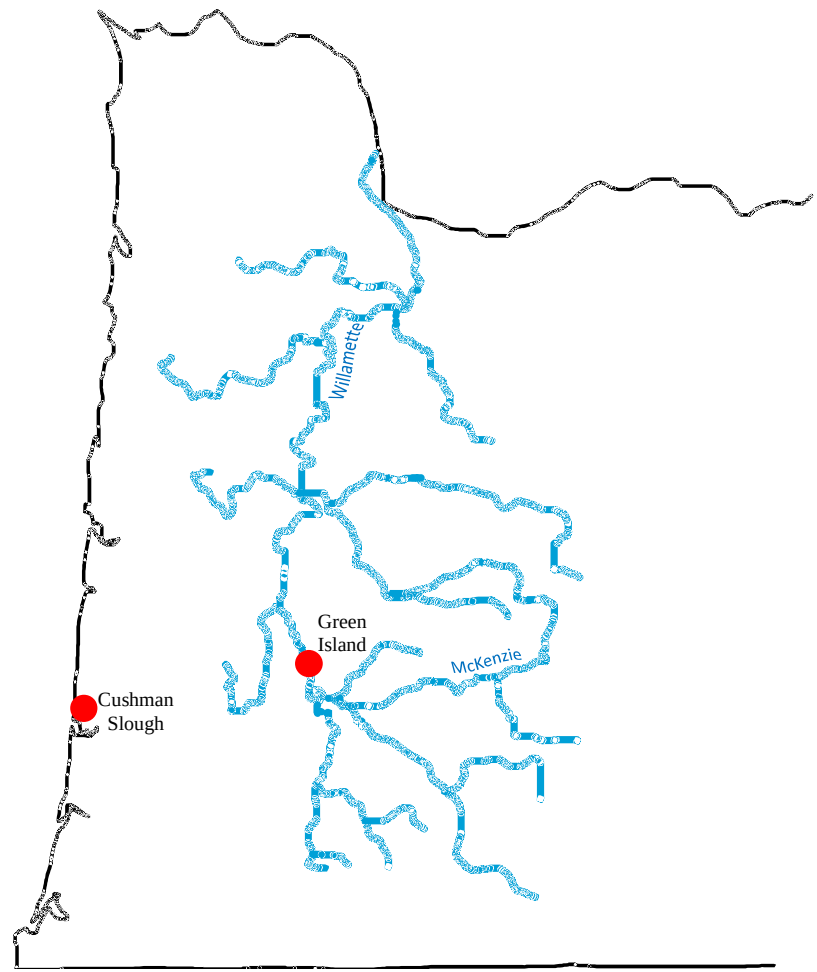
Figure 9: Map of major rivers in the Willamette Basin in Oregon. The collection sites for wild-caught parents are indicated for Cushman Slough and Green Island by red dots.

Table 4: Wild-caught individuals preserved for developmental time series

Population	Individuals preserved	Time Range
Cushman Slough	12	0 dph to 4 dph (except 2 dph)
Cushman Slough	39	0 dph to 12 dph (except 5 dph and 11 dph)
Green Island	12	0 dph to 3 dph
Green Island	21	3 dph, and 9 dph to 13 dph, and 16 dph
Green Island	30	4 dph to 12 dph, and 14 dph
Green Island	34	0 dph to 11 dph, and 17 dph
Green Island	38	0 dph to 8 dph,, 12 dph to 15 dph, and 18 dph
Green Island	35	13 dph to 24 dph

Finally, the effects of microbes on the development of the adaptive immune system can be assessed by creating gnotobiotic, or germ-free, embryos. Since the threespine stickleback develops within the chorion – the tough outer membrane surrounding the embryo – we can treat the eggs to remove microbes, and then raise the fish in a sterile environment until the yolk has been depleted. Methods have been developed in order to create germ-free fish and have been effectively used in threespine stickleback to study immune responses to microbiota (Milligan-Myhre *et al.* 2011; Milligan-Myhre *et al.* 2016).

With the knowledge of when the onset of the adaptive immune system occurs, we can analyze the onset of the adaptive immune system of fish who are not challenged by microbes and therefore may not develop adaptive immunity with the same timing.

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