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THE ESTUARINE KILLIFISH, Fundulus heteroclitus, AS A MODEL SYSTEM FOR DEVELOPMENTAL IMMUNOTOXICOLOGY

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THE ESTUARINE KILLIFISH, Fundulus heteroclitus, AS A MODEL SYSTEM FOR DEVELOPMENTAL IMMUNOTOXICOLOGY

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

by
Laura Renee Hunt
May 2007

Accepted by:
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ABSTRACT

Little is known about the development of the immune system in estuarine teleosts. The aim of this study was to describe the disappearance of maternally-derived immune components of immunity and the development of the immune system in the estuarine killifish, *Fundulus heteroclitus*, also known as the mummichog. Maternal-derived IgM was measured by ELISA and whole body lysozyme levels were determined by both enzymatic and immunological methods. Recombination activation gene-1 (*Rag-1*) expression was quantified using real time quantitative PCR and organ development was monitored using histological techniques. *Rag-1* was cloned from *F. heteroclitus* and a recombinant *Rag-1* protein was expressed for the production of *Rag-1* antibodies. *Rag-1* was expressed most in the head kidney, followed by the heart and brain. Using a capture ELISA, IgM was first detected at 1 day post-fertilization (dpf), but decreased shortly after hatching and did not increase for the remainder of the study period. Lysozyme protein was first detected at 7 dpf by immunoblot and IHC using a monoclonal antibody (mAb M24-2) specific to mummichog lysozyme. Expression levels of *Rag-1* increased rapidly at 2-wph reaching a peak at 3-wph. The head kidney was the first to appear at 7-dpf, followed by the spleen at 1-wph, and the thymus at 3-wph. These findings indicate that the mummichog has all lymphoid organs by 5-wph. However, they do not have aspects of humoral immunity until sometime after 5-wph, and thus probably rely on innate and passive immunity for at least 5-wph.

To the author’s knowledge, this study is the first to describe the ontogeny of the immune system in an estuarine teleost fish. This study will add to the growing body of information on the immuobiology of fishes. Moreover, this research provides baseline data for future studies on impacts of environmental stressors on developing fish immune systems.
DEDICATION

I dedicate this dissertation to my friends and family. This dissertation exists because of their love and support.
ACKNOWLEDGMENTS

I would like to acknowledge the following people for their support and contribution throughout graduate school. Dr. Charles Rice, my major professor, for his guidance throughout my entire graduate experience. I have learned a great deal about immunology and life from him. I appreciate his encouragement, support, and faith in me. My committee: Dr. Steven E. Ellis, Dr. Thomas Scott, Dr. Thomas Schwedler, and Dr. Peter Van den Hurk. Each provided invaluable advice and support throughout my research. My current and former labmates: Dr. Marlee Marsh, Abigail Babcock, Shannon Billings, Dr. Lee Ann Frederick, Dr. Alison Becker, and Dr. Rod Regala. None of my accomplishments would be possible without them. They have been there for me every step of the way, through blood, sweat, and tears, to the very end. I will forever cherish their friendship and am privileged to have them as colleagues. My fellow graduate students, Christy Geraci, Dr. Donna Abernathy, Dr. Alicia Manfre, Dr. Shala Hankison, Michele Kittel, Adrianna Zito, Dr. Rick Laughlin, Kristen Gaworecki, and Brittany Turner. I am very fortunate for their friendship. The entire department of Biological Sciences, working there has been an incredible experience. I would like to thank Dr. Matt Turnbull, Dr. John Wourms, Dr. Margaret Ptacek, Dr. Bert Abbott, Dr. Linda Gahan, and Jeremy Chambers for their guidance. Also, I give many thanks to Dr. Andy Mount, for not minding me dropping by for many engaging conversations. Finally, I would like to thank Corey Roelke for being there when I needed him most.
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Teleost lymphoid organs

The lymphoid organs in teleostean fish are the anterior kidney, thymus, spleen, and mucosal associated tissues (MALT) (Iwama and Nakanishi, 1996). The primary organs are the kidney, the main organ for B lymphocytes (Breuil et al., 1997) and the thymus, the main organ for T lymphocytes (dos Santos et al., 2000), while the spleen and mucosal associated tissues are considered secondary lymphoid organs (Press, 1999). The most notable difference between mammalian and teleost lymphoid organs is that teleostean fish lack bone marrow and lymph nodes (Zapata, 1979a).

The teleostean kidney is a complex multifunctional organ that has hematopoietic, reticuloendothelial, endocrine, and excretory functions (Zapata and Cooper, 1990). However, in many teleosts, the kidney is typically divided into two regions, an anterior portion, or head kidney, and a posterior, or trunk kidney. The distribution of lymphocytes within the teleostean head kidney can be variable in that lymphocytes have been observed in defined clusters (Zapata, 1979a) or scattered at random (Press et al., 1994) in the kidney parenchyma. There is substantial evidence that the head kidney is primarily hematopoietic in function, with some renal function, while the trunk kidney is mostly renal (Kobayashi et al., 2006; Willett et al., 1999; Zapata, 1979a). In trout, B cell subsets in the head kidney are very different from the subset pool in the posterior kidney (Zwollo et al., 2005).
The teleostean thymus is located near the gills opercular cavity and is often closely associated with the pharyngeal epithelium (Romano et al., 1999; O’Neill, 1989), and typically lacks clear corticomedullarly regionalization (Iwama and Nakanishi, 1996; Willett et al., 1997). As with the mammalian thymus, the teleost thymus is a site for T cell positive/negative selection and maturation (Abelli et al., 1998; Lam et al., 2002). In zebrafish, this is evidenced by the expression of TCRα and Rag-1, where TCR was expressed throughout the thymus, and Rag-1 was localized to peripheral regions (Danilova et al., 2004). Similarly, positive and negative selection in carp occurs in the cortex by four weeks post-fertilization (Romano et al., 1999).

Mature lymphocytes leave their primary lymphoid tissues and migrate to peripheral lymphoid organs. Lymphocytes will continue to recirculate between the lymph, blood, and lymphoid tissues until interaction with antigen or death (Janeway et al., 2001). In higher vertebrates, the spleen is a major peripheral lymphoid organ that functions to filter blood, trap and process antigens. The mammalian spleen can be divided into two regions, the red pulp, a site for erythrocyte disposal, and the white pulp, which resembles lymphoid nodules (Martini et al., 2001). The white pulp is comprised of periarteriolar lymphoid sheath (PALS), which contains mostly T cells, and the bordering B-cell corona, which contains mostly B cells (Janeway et al., 2001). The B cell corona and PALS are sites of antigen driven immune activation in the spleen, involving B cells, T cells, and antigen presenting cells (Liu, 1997). In teleosts, the spleen is also occasionally divided into a red and white pulp region (Iwama and Nakanishi, 1996; Falk et al., 1995; Mulero et al., 2006; Petrie-Hanson and Ainsworth, 2001). In contrast, fish do not have germinal centers, but are thought to
have equivalent structures called melanomacrophage centers (MMCs) (Herraez and Zapata, 1991; Iwama and Nakanishi, 1996; Vigliano et al., 2006). The putative functions of MMCs are to trap and process antigen (Herraez and Zapata, 1987; 1991; Meseguer et al., 1994), however, this remains a subject of debate. Nevertheless, MMCs can be found in the spleen, kidney, and liver of most teleosts (Passantino et al., 2005).

In fish, the mucosal immune system includes gut-associated lymphoid tissue (GALT), gills, and mucosal surfaces that cover the skin (Dalmo et al., 1997; Press, 1999). The primary function of the mucosal lymphoid tissues is to maintain immune defense where pathogen exposure is high. The mucosal surfaces are bathed in an array of antimicrobial molecules that are components of the innate immune system. Some examples of antimicrobial molecules are pleurocidin, isolated from winter flounder (Cole et al., 1997) and parasin I, isolated from catfish (Cho et al., 2002). In addition, lysozyme, an important antimicrobial protein, has been detected in a wide variety of teleosts (Lie et al., 1989; Murray and Fletcher, 1976). In addition to defense molecules, the mucosal tissues are also infiltrated by lymphocytes and neutrophils (Hebert et al., 2002).

**Ontogeny of the teleost immune system**

It is thought that B cells of teleostean fish mature in the head kidney and then migrate to peripheral sites of activation such as the spleen (Zwollo et al., 2005). In general, lymphoid organs appear prior to the appearance of immunocompetent immune cells. In mammals, the cells of the immune system are formed in the bone marrow from a common pluripotent hematopoietic stem cell. These stem cells then
differentiate into the myeloid and lymphoid compartments, which either mature in the bone marrow or migrate to peripheral tissues for maturation (Janeway et al., 2001). Teleost fish do not possess bone marrow. Instead, blood cells form within a hematopoietic kidney (Kobayashi et al., 2006; Zapata, 1979a; Zwollo et al., 2005).

The development of the thymus involves all three embryonic germ layers (Manley, 2000). In mammals, the thymus forms from the endoderm of the third pharyngeal pouch and the neural crest mesenchyme of the third and fourth pharyngeal pouches (Manley, 2000). In contrast, the fish thymus usually forms from the second, third, and fourth pharyngeal pouches (Bowden et al., 2005).

Fish hatch into a potentially pathogen rich environment with little immunocompetence. Thus, larval fish must have compensatory mechanisms to ensure survivability at vulnerable life stages. Fish embryos may receive some protection from maternally derived molecules, such as antibodies or lysozyme (Bly et al., 1986; Suzuki et al., 1994; Yousif et al., 1991; 1994; 1995), or they may rely on innate immunity until acquired immunity develops. To date, lymphoid ontogeny has been studied in select fish species including zebrafish (Lam et al., 2004), carp (Huttenhuis et al., 2005), channel catfish (Petrie-Hanson and Ainsworth, 2001), rainbow trout (Tatner, 1986; Tatner and Manning, 1983), sea bass (Breuil et al., 1997), and Atlantic cod (Schroder et al., 1998).

Although most fish have similar lymphoid organs, the onset of immune maturation and progression can differ among species (Zapata et al., 2006), particularly between freshwater and marine species. In freshwater teleosts, such as zebrafish, the thymus is first to become lymphoid, followed by kidney and then the spleen. In marine teleosts, such as Atlantic cod, the kidney is typically the first to
become lymphoid, followed by spleen, and ultimately the thymus (Zapata et al., 2006). The timing and sequence of lymphoid development could be dependent upon various environmental cues. For example, the immune system, as with other physiological systems, can be influenced by abiotic factors such as salinity (Cuesta et al., 2005), temperature (Engelsma et al., 2003; Guz and Kozinska, 2003), seasonality (Nakanishi, 1986; dos Santos et al., 2000), and spawning cycles (Nakanishi, 1986). To date, the influence of spawning strategy on the progression of immune development in fish has not been investigated.

**Introduction to the mummichog**

The mummichog, *Fundulus heteroclitus*, is distributed from Newfoundland to Florida (Bigelow and Schroeder, 1953) and is one of the most abundant species in high marsh habitats. These fish comprise a large percent of total secondary productivity in high marsh habitats (Meredith and Lotrich, 1979). Mummichogs prefer the high marsh habitat and subsequently have high home range fidelity throughout their life cycle (Kneib, 1986). Furthermore, mummichogs are relatively easy to maintain and culture in captivity and thus are ideal for studying environmental physiology in estuarine fish (Kneib, 1986, DiMichele et al., 1986), genetic diversity (Powers et al., 1986), and the effects of pollutants on estuarine fish (Weis et al., 1981; Gaworecki et al., 2004; Hahn et al., 2004; Meyer et al., 2003; van den Hurk et al., 2000; Wassenberg and Di Giulio, 2004; Wassenberg and Giulio, 2004; Wassenberg et al., 2005; Wassenberg et al., 2002). Lastly, mummichogs have large clear embryos and their normal stages of morphological development have been well documented (Armstrong and Child, 1965; Oppenheimer, 1937).
Mummichogs spawn according to lunar cues at the new and full moon during the reproductive season (Cochran et al., 1988). At the peak of the growing season, a gravid female can produce several hundred eggs in a single clutch (Armstrong and Child, 1965). The timing of organogenesis, especially lymphoid development and B- and T-cell maturation, may be critical to the survival of this high marsh fish. Whether or not maternally derived factors provide immunity prior to the onset of adaptive immunity is not known. Although there are descriptions of fish lymphoid organ development in other teleosts, the phylogenetic diversity and difference in early life history of fish make it difficult to construct comparisons. Furthermore, current fish models of immune development, such as zebrafish, are not environmentally relevant to the eastern shores of the United States. Therefore, identifying stages of lymphoid development in an estuarine fish, such as mummichog, could be useful for environmental studies along the East Coast of the USA.

Research Objectives

The aim of the present study was to describe the disappearance of maternally-derived IgM, and the appearance of autologous attributes of immunity, including lymphoid development in the estuarine killifish, Fundulus heteroclitus from fertilization through 5 weeks post-hatch.

Research Objective I

The goal of objective I was to clone Rag-1 from mummichog, describe Rag-1 gene expression in different tissues, generate anti-mummichog Rag-1 antibodies, and explore the use of this antibody for research on lymphoid ontogeny in fish.
Research Objective II

The goal of objective II was to identify the onset of lymphoid development in the mummichog by observing the disappearance of maternally-derived attributes of immunity and the appearance of autologous lysozyme and tissue Rag-1 expression, as well as the appearance and sequence of lymphoid tissues.

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CHAPTER 1

CLONING AND EXPRESSION OF RECOMBINASE ACTIVATION GENE (RAG-1) IN MUMMICHOG, FUNDULUS HETEROCLITUS

Abstract

The recombination activation genes, Rag-1 and Rag-2, encode for recombinases involved in V (D) J recombination of immunoglobulin and T-cell receptor genes. These genes are synchronously expressed during lymphocyte development and as such are useful markers for lymphoid ontogeny. A partial sequence of Rag-1 was cloned from mummichog, Fundulus heteroclitus, genomic DNA using degenerate primers. New specific primers were designed and used to amplify Rag-1 from cDNA for gene and protein expression analysis. Expression of Rag-1 gene in adult mummichog tissues was highest in the head kidney, followed by the heart, brain, and spleen. For protein expression analysis, a recombinant Rag-1 protein was generated for the production of anti-Rag-1 antibodies. Rag-1 protein expression was observed in adult mummichog head kidney leukocytes using the anti-Rag-1 antibody, which is a consistent finding in teleostean fishes, thus substantiating that the head kidney is a primary site for lymphopoiesis.
1.0 Introduction

The generation of diversity in T- and B-cell receptors (TCR, BCR) is the result of coordinated events in the maturation of lymphoid precursors, the most important of which is rearrangement of immunoglobulin (Ig) genes in B-cells, and TCR genes of T-cells. Successful rearrangement of Ig and TCR genes then serves as control steps for further maturation steps (Huang and Kanagawa, 2004; Reichman-Fried et al., 1990; Spanopoulou et al., 1994; Young et al., 1994). The genes for BCRs and TCRs encode multiple variable (V) regions, junctional regions (J), as well as diversity (D) regions that combine with constant (C) regions (Du Pasquier, 2004). The mature B- or T-cell expresses a receptor containing only one VJC- or VDJC-gene combination out of all the possible regions encoded in the genome (Oettinger et al., 1990). Recombination is dependent on recombinases and DNA-repair enzymes, but two recombinase genes are absolute requirements; recombination activation gene-1 (Rag-1) and recombination activation gene-2 (Rag-2). The Rag-1 and Rag-2 genes encode for components of the recombinase which catalyzes the recombination of multiple V, D, J heavy and V, -J light chain gene segments of Ig genes, and α:β and δ:γ chains of the TCR (Oettinger et al., 1990). Rag-1 and Rag-2 enzymes recognize recombination signaling sequences on the 5′ – regions D, and 3′-regions of V and J regions, resulting in the production of a large repertoire of TCR and Ig proteins that can recognize a multitude of antigens (Tonegawa, 1983).

The amino acid sequence of Rag-1 and -2, especially Rag-1, is highly conserved among vertebrates (Greenhalgh et al., 1993; Hansen and Kaattari, 1995; 1996; Oettinger et al., 1990; Willett et al., 1997). In Rag-1, the homology of the sequence is highest in the core region (Sadofsky et al., 1993; Willett et al., 1997),
which is also responsible for target site recognition (Sadofsky et al., 1995). The two Rag proteins work as a heterodimer and are synchronously expressed in the early stages of lymphocyte development (Sadofsky, 2001). An absence of either Rag gene results in a lack of mature B and T cells, which subsequently causes severe immune disorders such as severe combined immunodeficiency (SCID) (Mombaerts et al., 1992; Sobacchi et al., 2006). These findings provide additional support that the Rag genes are essential for proper immune development. Therefore, the expression of Rag-1 and -2 can be used to identify the appearance and location of lymphoid cells and organs during development of the immune system.

To date, most studies that examine lymphoid development in teleostean fish have used fish common to aquaculture/mariculture or genetic research such as the channel catfish (Petrie-Hanson and Ainsworth, 2001), rainbow trout (Castillo et al., 1993; Tatner, 1986; Tatner and Manning, 1983), zebra fish (Lam et al., 2004; Lam et al., 2002), and sea bass (Breuil et al., 1997; dos Santos et al., 2000). The mummichog, Fundulus heteroclitus, is a unique model for studies on developmental immunology because it is euryhaline, has a high degree of thermal tolerance, and has a unique reproductive strategy (Bigelow and Schroeder, 1953). Mummichogs inhabit the high marsh habitat of estuaries along the east coast of North America, and as such are subjected to rapid changes in daily, lunar, and seasonal salinity and temperature. As an adaptation to their environment, mummichogs reproduce on the lunar cycle throughout the warmer months of the year (Hsiao et al., 1994). At the spring high tide, adult mummichogs deposit fertilized eggs in the high marsh, which desiccate until the subsequent spring high tide on the next lunar cycle. Re-hydration initiates development of the embryos that wash out to the marsh on the outgoing tide.
In the study described herein, a partial mummichog Rag-1 sequence was cloned and sequenced, and shown to be highly homologous to Rag-1 genes of multiple vertebrates. Gene expression analysis shows that head kidney is the primary location for Rag-1 gene expression, though both heart and brain tissues express the gene. For protein expression analysis, a recombinant Rag-1 protein (rRag-1) was generated for the production of anti-Rag-1 antibodies. Immunofluorescence using the anti-rRag-1 protein antibody shows that the protein is abundantly expressed in head kidney cells of adult mummichogs.

2.0 Materials and Methods

2.1 Animals

Adult mummichogs, Fundulus heteroclitus, were collected at the Belle W. Baruch Marine Lab, Georgetown SC, USA, on the full and new moon using baited minnow traps. Some adult fish were transported to laboratory facilities at Clemson University in aerated seawater and transferred to holding facilities containing 18 parts per thousand (ppt) artificial seawater (Instant Ocean, Pet Warehouse). Adult fish were maintained at 22 - 27 °C and fed Tetramin flake food (Tetra Sales) twice daily. At the time of collection, select male and female fish were stripped for milt and eggs according to procedures first described by Armstrong and Child (1965). Briefly, oocytes were manually stripped from gravid females and fertilized in vitro using milt manually collected from spawning males. Fertilized eggs were transported to the laboratory in ambient seawater and transferred to glass Petri dishes. Non-viable eggs were then removed by aspiration with aid of a dissecting microscope. Embryos were
monitored for stage-specific development and, once hatched, larval fish were transferred to grow-out tanks and fed live *Artemia* once a day.

### 2.2 Cloning mummichog Rag-1

A portion of mummichog Rag-1 was obtained from polymerase chain reaction (PCR) amplification of mummichog genomic DNA using degenerate primers (Table 1) according to previously published methods (Greenhalgh *et al*., 1993). The Rag-1 sequence is highly conserved among vertebrates, therefore degenerate primers were designed from an alignment of amino acid sequences from human, mouse, and chicken Rag-1 (Greenhalgh *et al*., 1993). For genomic DNA extraction, the liver was removed from one euthanized (MS-222, 1 g/L) adult animal. DNA was isolated using the Wizard® genomic DNA purification kit according to manufacturer’s protocol (Promega). A 50 µl PCR reaction was performed with the Accuprime Taq polymerase system (Invitrogen). The reaction was cycled in a Perkin-Elmer thermocycler using the following conditions: initial denaturation at 95°C for 5 min, 30x [95°C 1 min, 50°C 1 min, 72°C 2 min], and final extension at 72°C for 10 min. The expected fragment size of ~600 bp was visualized on a 1% agarose gel. The product was cloned into the pCR®2.1 vector using the TA cloning kit (Invitrogen) and positive clones were selected through PCR screening. Positive clones were digested with *EcoRI* (Promega) for further validation and then sequenced at the University of Maine Sequencing Facility (Orono, ME).
2.3 Oligonucleotides for cloning

All primers for cloning are summarized in Table 1.1. Degenerate primers to *Rag-1* were initially used to amplify *Rag-1*, and after sequencing to confirm identity, new specific primers were produced. For cloning into the expression construct, *Rag-1* primers with Sac I and Hind III restriction sites were designed. The *Rag-1* forward primer contained the Sac I restriction site while the reverse primer contained the Hind III restriction site. To aid restriction enzyme binding to the restriction site sequence, four extra nucleotides were included at the end of each restriction site sequence. All oligonucleotides were synthesized at Sigma-Genosys (Sigma).

2.4 cDNA construct

Total RNA was isolated from developing mummichogs for production of cDNA. In early development it is not possible to isolate organs, therefore, whole animals were homogenized. Total RNA was isolated from developing mummichog embryos at 1 week post-fertilization using a micro-bead homogenizer (Biospec Products, Inc.) with 2 mm zirconia beads and TriReagent® (100 µl per mg tissue) using the manufacturers directions. RNA purity was determined with a UV spectrophotometer (Eppendorf) at OD_{260/280} and a ratio > 1.7 was accepted for further reactions. To eliminate possible genomic DNA contamination, 1 µg of total RNA was treated with amplification grade DNase I (Invitrogen) prior to reverse transcription. For cDNA, total RNA (1 µg) was primed with oligo-(dt)$_{12-18}$ and reverse-transcribed with SuperScript™ (Invitrogen), as described by the manufacturer. All cDNA samples were stored at -20°C until further analysis.
For the cDNA construct, new gene specific primers (GSP) were designed from a portion of mummichog genomic \textit{Rag-1} (720 bp) previously amplified with degenerate primers. \textit{Rag-1} was amplified from cDNA with 5 \(\mu\)M of each GSP using Accuprime system (Invitrogen) following the manufacturer’s protocol. The reactions were run at the following conditions: 95\(^\circ\)C for 2 minutes, 25x \([95\, ^\circ\, C \ 30\, s,\ 50\, ^\circ\, C \ 30\, s,\ 72\, ^\circ\, C \ 30\, s]\), and final extension at 72\(^\circ\)C for 7 minutes.

The PCR product was cloned into the pCR\textsuperscript{®}2.1 vector using the TA cloning kit (Invitrogen) and positive clones were selected through PCR screening. Positive clones were digested with \textit{EcoRI} (Promega) for further validation and then sequenced at the University of Maine Sequencing Facility (Orono, ME). The deduced amino acid sequence from cDNA was then compared to zebrafish, mouse, human, rainbow trout, and \textit{Xenopus} \textit{Rag-1} sequences using the alignment software ClustalW (Chenna \textit{et al}., 2003).

\subsection*{2.5 Expression construct}

Recombination activation gene 1 cDNA was used to generate a recombinant \textit{Rag-1} protein. For protein expression, \textit{Rag-1} cDNA was ligated into the pQE-32 expression vector (Qiagen) in-frame with the six-histidine (HIS) residue N-terminal sequence. Briefly, a 385 bp sequence from the cDNA construct was amplified with restriction sites on each end of the sequence. The restriction sites, Sac I and Hind III, were used to directionally clone the sequence into the expression vector. Following ligation, the construct was subcloned into XL-2 blue super competent strain of \textit{E. coli} (Stratagene) and sequenced to verify reading frame.
2.6 Recombinant protein expression

For protein expression, pQE-32 was isolated from XL-2 Blue cells by mini-prep then transformed into ArcticExpress™ (DE3) competent cells (Stratagene). The DE3 cells co-express the cold-adapted chaperons Cpn10 and Cpn60 from Oleispira antartica, thus conferring improved protein processing at lower temperatures, which increases the amount of soluble recombinant protein produced. Following the manufacturer’s protocol, transformed ArcticExpress™ cells were grown on LB agar plates with 100 µg/ml ampicillin overnight. The next day, 1 ml LB broth containing 100 µg/ml ampicillin and 20 µg/ml gentamycin were inoculated with overnight colonies and grown overnight. These cultures were subcultured into LB broth containing no selection antibiotics and protein expression was induced with 1mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 10-13°C for 24 hrs. Prior to induction, a 1 ml sample was collected as the non-induced control.

After induction, cells were collected by centrifugation at 10,000 xg for 30 minutes and the supernatant was discarded. The pellet was resuspended in 10 ml of distilled water, gently mixed with lysis buffer [2 ml DI water, 2 ml 1 M Na₂HPO₄, 0.2 ml 100 mM PMSF, 2 ml 10% Triton X-100, 0.6 ml 5 M NaCl, and 0.04 ml 10 M NaOH] for 15 minutes on an orbital rotor, and then subjected to 3 freeze thaw cycles in liquid nitrogen. After lysis, the mixture was centrifuged at 10,000 xg for 30 minutes to obtain the soluble supernatant fraction and an insoluble pellet, which was discarded.
2.7 Protein purification

Histidine-tagged recombinant Rag-1 was purified from the soluble fraction by incubating the fraction with 100 µl of nickel-nitrilotriacetic acid (Ni-NTA) agarose (Qiagen) for 15 minutes with rocking at room temperature. The agarose was centrifuged at 1,375 x g for 5 minutes. Initially, small cultures (50 ml) were screened for the presence of His-tagged proteins. For larger quantities of recombinant protein, cleared lysates from large cultures (1L) were bound to Ni-NTA and loaded onto a disposable polystyrene column. The agarose was resuspended in 0.25 ml wash buffer (50 mM Tris, 300 mM NaCl, 25 mM imidazole, 10% glycerol, pH 6.8), loaded onto a disposable polystyrene column (Pierce), and washed an additional 3 times with 0.5 ml of wash buffer. Purified protein was eluted with low pH buffer (50 mM Tris, 300 mM NaCl, 200 mM imidazole, 10% glycerol, pH 6.8) and quantified with the bicinchoninic acid assay (BioRad). Protein from non-induced control, washes, and elutions were separated on 4-20% SDS-PAGE gels (BioRad) via electrophoresis (PAGE). The recombinant Rag-1 protein expressed from the pQE-32 vector is ~21 kDa in size.

2.8 Immunization and generation of antisera

Antisera against recombinant Rag-1 protein was generated in 6-8 wk old female RBF/dnj mice (Jackson Labs) using immunization procedures as described by (Rice et al., 1998). Following booster immunizations, mice were euthanized and bled for serum collection. Serum samples were stored at –20º C until further use.
2.9 Preparation of tissues for Rag-1 gene and protein expression analysis

To determine tissue RNA gene expression, four adult mummichogs were euthanized in 1 g/L MS-222 and brain, heart, gill, anterior kidney, spleen, intestine, and gonads were quickly pooled per tissue and homogenized in TriReagent® (Molecular Research Center, Inc.) per the manufacturer’s protocol. Homogenized samples were stored in TriReagent® at -80°C until further analysis.

2.10 Isolation of total RNA and QT-PCR for Rag-1 gene expression in mummichog tissues

Total RNA was isolated with TriReagent® at a concentration of 100 mg tissue/ml of TriReagent® according to the manufacturer’s protocol. RNA purity was determined with a UV spectrophotometer (Eppendorf) at OD_{260/280} and a ratio > 1.7 was accepted for further reactions. Tissue Rag-1 quantitative expression profiles were generated with the iCycler real-time PCR system (BioRad). One microgram of total RNA from each of the pooled tissues was DNase I-digested, reverse transcribed with oligo (dT) as primer and equivalent aliquots of first strand cDNA were then amplified using respective sets of primers (Table 1.2) with the following conditions: initial denaturation at 95°C, 40 cycles of denaturation at 95°C for 30 s and annealing at 65°C for 30 s, followed by a final extension at 55°C for 1 minutes. Melt curve analysis was performed with continuous fluorescence from 65 to 95°C at a temperature transition rate of 0.01°C/s to determine amplification specificity. All oligonucleotides were synthesized by Sigma-Genosys (Sigma). Reactions were run in 96-well PCR plates (BioRad). Each sample was run in quadruplicate together with the appropriate non-template controls and known dilutions of plasmid cDNA, carrying a fragment of the respective target gene, ranging from 0.001, 0.0001,
0.00001, 0.000001, 0.0000001ng. For each run, a standard curve was generated for β-actin and Rag-1 using known concentrations of respective plasmid cDNA carrying a fragment of the respective target gene. The concentration of β-actin and Rag-1 in unknown samples was determined from the β-actin and Rag-1 standard curve, respectively. Rag-1 was normalized to β-actin by dividing ng of Rag-1 by ng of β-actin, previously generated from the standard curves. Quantitative results are represented as relative levels of Rag-1 normalized to β-actin. In all quantitative real-time PCR runs, melt curve analyses were performed and single peaks were observed indicating amplification specificity, and that non-template controls were negative (data not shown).

2.11 Construction of standard curve for quantitative real time PCR

Quantitation of real time PCR was according to the standard curve method using purified cDNA plasmid standards carrying a fragment of the respective target genes. The validity and suitability of utilizing cDNA plasmids for quantitation has been previously described (Giulietti et al., 2001). This method is advantageous because the standard curve serves as an additional control, reducing inter-assay variability (Giulietti et al., 2001). The endogenous housekeeping gene, β-actin, was used as reference in all quantitative real time PCR runs. β-actin has been previously utilized as a reference gene in F. heteroclitus in quantitative real time PCR (Roling et al., 2004) and RT-PCR (Powell et al., 2000). A mummichog β-actin cDNA plasmid containing 291 nucleotides (AY735154) of the coding region was cloned into the TA overhang vector pCR® 2.1 (Invitrogen). The Rag-1 cDNA plasmid contained 300 nucleotides (DQ250438) of the coding region in the pQE-32 vector (Qiagen). The
plasmids were purified via mini-prep (Promega) and the concentrations were
determined by OD_{260} measurements. Standard curves for β-actin and Rag-1 were
generated using known concentrations (0.001, 0.0001, 0.00001, 0.000001, 0.0000001ng.) of plasmid cDNA. All calculations and methods for generating the
standard curves were according to manufacturer’s protocol (Applied Biosystems).
Specifically, the log nanogram of known plasmid cDNA concentrations was plotted
against respective Ct values to generate a line graph. Finally, the amount of
unknown sample, in nanograms, was determined by solving for log nanogram with
the line equation.

2.12 Immunofluorescence of Rag-1 in mummichog leukocytes

Another batch of adult mummichogs were euthanized and their anterior
kidneys removed and pooled. Next, lymphoid cells were isolated, by gentle pushing
of the tissues through a 100-mesh screen in Hanks balanced salt solution (97 g NaCl,
4.89 g KCl, 12.2 g glucose, 0.733 g KH_{2}HPO_{4}, 1.1 g Na_{2}HPO_{4}H_{2}O in 1 L Di H_{2}O),
hereafter referred to as HBSS, using a syringe plunger. Cells in HBSS were
transferred to a 15 ml tube and the debris was allowed to settle by gravity for 3
minutes. The overlying cells were removed and washed twice with HBSS by
centrifugation. To reduce the number of red blood cells, the cells were centrifuged
over histopaque 1.119 (Sigma-Aldrich), collected, and washed by centrifugation.
Lymphoid cells were then suspended in HBSS and counted using a hemocytometer.
Cell viability was determined by trypan-blue exclusion.

To detect Rag-1 protein expression, lymphoid cells were first fixed with a 3%
solution of methanol-free formalin in HBSS for 30 minutes, and then washed twice
by centrifugation in HBSS. Cells were then permeabilized with a 0.1% solution of Triton-X 100 in HBSS for 10 minutes, washed twice with HBSS by centrifugation, and blocked with blocking buffer (3% BSA and 0.2% gelatin in HBSS) for 30 minutes. The cells were washed with HBSS by centrifugation, resuspended in HBSS at a density of $10^7$ cells per ml in 1 ml aliquots. Antisera against recombinant Rag-1 was added to the one ml of cells to a final dilution of 1:250, and the cells were rocked at room temperature for 1 hr. After the 1 hr incubation period, the cells were washed twice with HBSS by centrifugation and suspended in HBSS. Goat anti-mouse IgG conjugated with FITC (Southern Biotechnology) was added to the suspension to a final dilution of 1:300, while another set of cells were incubated with just secondary antibody as control. Cells were incubated at room temperature for 30 minutes, washed 3 times with HBSS by centrifugation, and suspended in HBSS. Cells were then examined for Rag-1 protein with a Zeiss LSM510 confocal microscope.

### 2.1.3 Immunoblot analysis of recombinant and native Rag-1 protein

For analysis of recombinant and native Rag-1 protein, known concentrations of protein samples were mixed with 4x sample buffer (40% glycerol, 240 mM Tris-Cl pH 6.8, 8% SDS, 1% bromophenol blue with 5% (v/v) β-mercaptoethanol), and heat denatured at 100°C for 7 minutes. Denatured proteins were resolved by SDS-PAGE (4-20%) and transferred to polyvinylidene fluoride (PVDF) membrane. A total of 1.2, 0.62, 0.31, and 0.13 µg of the recombinant protein was loaded in different lanes. For leukocyte Rag-1 protein analysis, the gel was loaded with 25 µg of total protein from mummichog head kidney leukocyte cell lysates. The
membranes were blocked for 1 hr with 3% bovine serum albumin (BSA) in phosphate buffered saline, pH 7.2 (PBS) containing 0.2% gelatin, hereafter referred to as blocking buffer, with rocking. Afterwards, the membranes were washed 3 times with PBS containing 0.5% Tween-20 (PBS-Tw20) and incubated for 1 hr with mouse anti-Rag-1 polyclonal antisera (diluted 1:1000 in PBS). Membranes were then washed 3 times with PBS-Tw20 and incubated for 30 minutes with alkaline phosphatase (AP) conjugated-goat anti mouse IgG-AP. Finally, membranes were washed 3 times with PBS-Tw20, and alkaline phosphatase activity visualized with 5-bromo-4-chloro-3-indoyl-phosphate/nitro-blue tetrazolium (NBT/BCIP) (Fisher Scientific) in 100 mM NaCl, 5 mM MgCl₂, 100 mM diethanolamine, pH 9.5 (AP buffer). Immunoblots were imaged with a GS-710 densitometer (BioRad).

3.0 Results and Discussion

The unique life-history strategy of the mummichog compared to other teleostean fish commonly used in comparative immunology begs the question of whether or not the development of the immune system of this animal deviates from what has been previously described. Answering such a question is important because the mummichog is routinely used in environmental research applicable to both environmental toxicology (risk assessment) and environmental physiology (Meyer et al., 2000; Radtke and Dean, 1979; Zhou et al., 2000). Moreover, because of the restricted home range of natural populations of mummichogs, these fish are good indicators of the health of the environment in which they reside. This is particularly relevant to immunology because several classes of environmental pollutants are known to be immunotoxic in a variety of both rodent and fish models.
Furthermore, since the mummichog is easily reared in laboratory settings and the embryological development of the animal is so well characterized (Armstrong and Child, 1965; Oppenheimer, 1937), answering questions relevant to mummichog immunology would be easy to address.

A drawback to studying fish immune systems is the general lack of molecular and immunological reagents with broad species cross-reactivity. As a result, comparative immunologists are forced to develop and characterize reagents on a species-by-species basis. The molecular and antibody-based tool box for studying mummichog immunobiology is growing, as can be seen at the website www.mummichog.org, in which newly sequenced genes and a list of available antibodies are posted. For example, mAb D58 recognizes IgM heavy chain, mAb 1C6 is specific for IgM light chains, and as described by Marsh (2007), mAb M24-2 is lysozyme specific, and mAb 2C11 is specific for a granule protein found only in highly granular leukocytes that are probably eosinophilic granulocytes.

The purpose of the study described herein was to develop new reagents for studying Rag-1 expression in mummichogs, and possibly additional species if these reagents are ultimately found to cross-react in other fish systems. Using degenerate primers, a 639 nucleotide portion of mummichog Rag-1 was cloned from the genomic DNA and sequenced (Fig. 1.1). The mummichog Rag-1 genomic DNA sequence has been deposited in the GenBank database under accession number DQ250438. Using specific primers, the cDNA sequence was obtained and sequenced, which encodes a protein of ~15 kDa in size (Fig. 1.2). The deduced amino acid sequence for mummichog Rag-1 cDNA was aligned with known Rag-1 sequences from zebrafish, rainbow trout, mouse, human, and Xenopus (Fig. 1.3), and
shown to be 86% identical to rainbow trout, 85% to zebrafish, 81% to human, 81% to mouse, and 78% African clawed frog, with the highest homology in the “core” region. Such a high degree of homology among vertebrates substantiates the theory that a key factor in the evolution of specific immunity (BCR and TCR) was the appearance of Rag-genes in early vertebrate lineages (Du Pasquier, 2004).

The similarity between mummichog Rag-1 protein sequence and that of rainbow trout, zebrafish, mouse, human, and Xenopus is primarily concentrated in the carboxyl-terminal two-thirds of the protein. The highly conserved carboxyl-terminal segment comprises the Rag-1 “core” region (Willett et al., 1997; Sadofsky et al., 1993). The core region is considered to be essential for recombination of artificial constructs in vitro (Sadofsky, 2001; Sadofsky et al., 1993; Sadofsky et al., 1995), which is responsible for nuclear targeting and DNA binding (Spanopoulou et al., 1995). The core region corresponds to amino acid residues 1-130 of the mummichog Rag-1 amino acid sequence.

Quantitative real time PCR analysis was performed on total RNA isolates from head kidney, gonads, gills, intestine, spleen, brain, and heart. Rag-1 expression was detected at the highest intensity in the head kidney, followed by heart, brain, and spleen, but not found in the other tissues (Fig. 1.4). These data support studies from other fish systems demonstrating the head kidney as the primary hematopoietic organ in teleostean fish (Huttenhuis et al., 2005; Iwama and Nakanishi, 1996; Lam et al., 2004; Willett et al., 1997). Rag-1 expression in brain has been demonstrated in other species, including the urodele amphibian (Frippiat et al., 2001), mice (Chun et al., 1991), and zebrafish (Feng et al., 2005; Jessen et al., 2001). There are several hypotheses for Rag-1 expression in nervous tissue, but the exact role is unclear.
Some speculate that it may be involved in odorant receptor rearrangement (Jessen et al., 2001) or learning and memory responses (Cushman et al., 2003). Though the heart is not typically considered a lymphoid organ in teleosts, lymphoid tissues have been observed covering the heart of sturgeons and paddlefish (Clawson et al., 1966; Peterman and Petrie-Hanson, 2006). The significance of Rag-1 expression in the non-lymphoid tissues of mummichog has yet to be determined.

Relevant to further developing the immunological tool box for understanding mummichog immunobiology, the development herein of protocols for quantifying Rag-1 expression offers the ability to follow expression in developing embryos and will lead to an understanding of the ontogeny of lymphoid development in the mummichog.

Ultimately, an anti-mummichog Rag-1 antibody may elucidate sites of Rag-1 protein expression during mummichog development but also sites that may be completely novel in mummichog. To generate antibodies against mummichog Rag-1, a recombinant protein was generated with the pQE-32 expression vector. Using a novel system that allows bacteria to express soluble recombinant protein at low temperatures, thus avoiding the formation of inclusion bodies that need denaturation steps, ~60 µg rRag-1 protein/ml of column elute was obtained. Separation of the proteins by electrophoresis and coomassie staining of the gel revealed that the purified recombinant Rag-1 protein was approximately 21 kDa in size and located primarily in the first and second elution (Fig. 1.5). Proteins of greater than 10 kDa are desirable for generation of specific antibodies (Harlow and Lane, 1999), therefore, the rRag-1 protein expressed in E.coli was used to immunize mice for the production of polyclonal antisera.
Native Rag-1 protein is approximately 121 kDa in zebrafish (NP571464) and rainbow trout (AAA80281), and approximately 119 kDa in mice (NP033045) and humans (NP000439). The resulting antisera recognized both recombinant protein (Fig. 1.6a) and a protein of the expected size of about 120 kDa in lysates of cells isolated from head kidney of adult mummichogs (Fig. 1.6b).

Leukocytes from adult mummichog head kidney were then isolated, fixed, and labeled with anti-Rag-1 polyclonal antibody. Approximately 50% of the cells appeared to be Rag-1 positive, with some cells having label predominantly in the nucleus (Fig. 1.7a), while the negative control displayed no fluorescence (Fig. 1.7b). These observations indicate that approximately 50% of the leukocyte population was positive for Rag-1 protein expression, which supports gene expression data from this study. With an antibody in hand against mummichog Rag-1 protein, investigators should be able to examine expression in a variety of tissues and cell types, and to determine the intracellular location of the protein. In addition, the development of quantitative real-time PCR for quantifying Rag-1 expression and the availability of specific antibodies will allow environmental scientists to quantify expression of this key protein in fish exposed to environmental pollutants known or suspected to be immunotoxic. Thus, Rag-1 expression should be a useful biomarker of immunocompetence in fish.
### Table 1.1
Oligonucleotide primer sequences for cloning mummichog *Rag-1*

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5'-3')</th>
<th>Tm C°</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Degenerate</td>
<td>CAYTGYGAYATHGGIAAYGC</td>
<td>55.3</td>
<td>638bp</td>
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<tr>
<td><em>Rag-1</em> Forward</td>
<td>TTRTGIGCRTTCATRAAYTTYTG</td>
<td>59.6</td>
<td>638bp</td>
</tr>
<tr>
<td>Degenerate</td>
<td>AACCAGTGATGAGGATG</td>
<td>53.5</td>
<td>379bp</td>
</tr>
<tr>
<td>GSP <em>Rag-1</em> Forward</td>
<td>ACTGTTCAAGCGGTTTCAG</td>
<td>61.5</td>
<td>379bp</td>
</tr>
<tr>
<td>Degenerate</td>
<td>GTACGAGCTCAACCAGTGATGAGGATG</td>
<td>71.3</td>
<td>385bp</td>
</tr>
<tr>
<td><em>Rag-1</em> Forward</td>
<td>ATCGAAGCTTGAAACCAGCGCTGAACAGT</td>
<td>75.0</td>
<td>385bp</td>
</tr>
</tbody>
</table>

*a* Degenerate sequence obtained from Greenhalgh *et al.* (Greenhalgh *et al.*, 1993). *(Y is C or T, R is A or G, H is A, C, or T)*

*b* Gene specific primers (GSP) for cDNA construct.

*c* Primers for expression construct. Restriction site portion of primer sequences are underlined
Table 1.2
Oligonucleotide primer sequences for quantitative real-time PCR

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequences (5'-3')</th>
<th>Tm C°</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rag-1 Forward&lt;sup&gt;a&lt;/sup&gt;</td>
<td>GGAACTACGCCCGAAGGTTAAT</td>
<td>56.7</td>
<td>131bp</td>
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<tr>
<td>Rag-1 Reverse&lt;sup&gt;a&lt;/sup&gt;</td>
<td>ACGCCACACAGGTTTCATCT</td>
<td>56.7</td>
<td>131bp</td>
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<tr>
<td>β-actin Forward&lt;sup&gt;b&lt;/sup&gt;</td>
<td>TATGCAGAAGGAGATCACTGCC</td>
<td>56.3</td>
<td>135bp</td>
</tr>
<tr>
<td>β-actin Reverse&lt;sup&gt;b&lt;/sup&gt;</td>
<td>ATCCACATCTGCTGGAAGGT</td>
<td>55.8</td>
<td>135bp</td>
</tr>
</tbody>
</table>
Fig. 1.1 Partial nucleotide and deduced amino acid sequences of *Fundulus heteroclitus* genomic Rag-1. The amino acid sequence is depicted in 1 letter code. The mummichog Rag-1 sequence has been deposited in the GenBank database under accession number DQ250438.
Fig. 1.2. Mummichog cDNA ORF sequence from start codon in pQE-32 expression vector with deduced amino acid sequence below. The mummichog \textit{Rag-1} cDNA sequence is highlighted in bold.
Fig. 1.3. Deduced amino acid sequence of mumichog, Fundulus heteroclitus, Rag-1 cDNA compared with the amino acid sequence of zebrafish, rainbow trout, mouse, human, and Xenopus Rag-1 using ClustalW (Chenna et al., 2003). (*) indicates identity with the mumichog sequence; (-) indicates gap in sequence; (;) indicates residues that are approximately the same size and the same hydropathy; (:) indicates where size or hydropathy has been conserved. The mumichog sequence is highlighted in bold.
Fig. 1.4. Quantitative expression profile of *Rag-1*, in head kidney, spleen, heart, brain, intestines, and gills generated from the iCycler real-time PCR system. The *Rag-1* expression profile shows the highest intensity in head kidney, followed by heart, brain, and spleen. One microgram of total RNA from each tissue was DNase I digested, reverse transcribed with oligo (dT) as primer and equivalent aliquots of first strand cDNA were then amplified (40 cycles) using respective sets of primers by the iCycler real-time system. Each sample was run in quadruplicate together with the appropriate non-template controls and known dilutions of the respective plasmid cDNA, carrying a fragment of the respective target gene, ranging from 0.001, 0.0001, 0.00001, 0.0000001ng. Standard curves for *β-actin* and *Rag-1* were generated using known concentrations (0.001, 0.0001, 0.00001, 0.0000001ng) of plasmid cDNA. To minimize variability due to differences in RT efficiency and RNA quality, data were normalized, by dividing ng of target cDNA by ng of *β-actin*, the endogenous housekeeping gene. Results are reported as relative levels of *Rag-1* expression normalized to *β-actin*. In all quantitative real-time PCR runs, melt curve analyses were performed and single peaks were observed indicating amplification specificity, and that non-template controls were negative (data not shown). Each tissue profile represents tissues pooled from four individual fish.
Fig. 1.5. Purified *E.coli* expressed Rag-1 protein. Hexahistidine tagged mummichog Rag-1 N-terminal protein were expressed in *E.coli* and purified on Ni-NTA agarose loaded columns. Wash and eluted proteins (denatured in 4x sample buffer) were separated on a 4-20% Tris-HCL SDS polyacrylamide gel and stained with coomassie brilliant blue. Right from kDa marker, wash 1 (lane 1), wash 2 (lane 2), wash 3 (lane 3), wash 4 (lane 4), elution 1 (lane 5), elution 2 (lane 6). Separation of the proteins by electrophoresis and coomassie staining of the gel revealed that the purified recombinant Rag-1 protein was approximately 21 kDa in size and located primarily in the first and second elution (in box).
Fig. 1.6a. Mouse antisera specifically recognized the target recombinant Rag-1 at 1.2 (Lane 1), 0.62 (Lane 2), 0.31 (Lane 3), and 0.13 (Lane 4) µg at ~21 kDa, the putative size of the recombinant Rag-1 protein. Recombinant protein was purified over Ni-NTA agarose and resolved on 4-20% Tris-SDS Polyacrylamide gel and immunoblotted. Immunoblots were probed with raw antisera at a working dilution of 1:1000 in phosphate buffered saline pH (PBS). From the start codon in the pQE-32 expression vector, the recombinant protein is approximately 21 kDa.
Fig. 1.6b. Mouse antisera specifically recognized leukocyte Rag-1 protein, at approximately 120 kDa, isolated from head kidney leukocyte lysates. Right to left: marker (Lane 1), recombinant Rag-1 protein at 1.2 µg (Lane 2), and total protein from head kidney lysates at 6.25 (Lane 2), 12 (Lane 3), 25 µg (Lane 4). Lysates were resolved on a 10% Tris-SDS Polyacrylamide gel and immunoblotted. Immunoblots were probed with antisera at a working dilution of 1:1000 in PBS. The putative size of leukocyte mummichog Rag-1 protein and recombinant Rag-1 is approximately 120 kDa (in box) and 21 kDa (Lane 2), respectively.
Fig. 1.7a. Two channel confocal laser scanning microscopy of leukocytes isolated from mummichog, Fundulus heteroclitus, head kidney. (A) Fluorescence channel of leukocytes labeled with anti-Rag-1 polyclonal antibody (B) Differential interference contrast (DIC) channel of same cells (C) Overlay of fluorescence and DIC channels.
Fig. 1.7b. Two channel confocal laser scanning microscopy of leukocytes isolated from mummichog, *Fundulus heteroclitus*, head kidney. (A) Fluorescence channel of leukocytes labeled with secondary antibody only as control (B) Differential interference contrast (DIC) channel of same cells (C) Overlay of fluorescence and DIC channels
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CHAPTER 2

THE DISAPPEARANCE OF MATERNALLY-DERIVED ATTRIBUTES OF IMMUNITY AND APPEARANCE OF AUTOLOGOUS COMPONENTS OF IMMUNITY IN DEVELOPING MUMMICHOG, *FUNDULUS HETEROCLOITUS*

Abstract

The development of the immune system in mummichog was investigated using a combination of enzyme-linked immunosorbent assays (ELISA), immunoblotting techniques, and immunohistochemistry (IHC). Embryonic and larval mummichogs were sampled at 1-, 2-, 3-, 4-, 7- day post-fertilization (dpf), 1 day post-hatch (dph), 1-, 2-, 3-, 4-, 5- weeks post-hatch (wph). Using a capture ELISA, IgM was detected in the embryonic stage (1-7dpf), but dropped precipitously after hatching and did not increase for the duration of the study period. Lysozyme activity rose rapidly after hatching and remained relatively high until the end of the study period. Lysozyme protein was detected at 7-dpf, 1-dph, at 1-wph, decreased slightly at 2-wph, and then gradually increased from 3- to 5-wph. *Rag-1* expression first increased at 2-wph, reached a maximum intensity at 3-wph, and then dropped to pre-2 wph levels for the duration of the study period. The first lymphoid organ to appear was the head kidney, followed by the spleen and then the thymus. Cells positive for lysozyme protein were first detected at 7-dpf and continued to increase in prevalence and distribution for the duration of the study period. Overall, this
study suggests that juvenile mummichogs have aspects of innate immunity but do not possess acquired immunity until some time after 5-wph.

1.0 Introduction

Currently, there is little information on immune capabilities of juvenile fish, particularly in euryhaline and thermally-tolerant estuarine teleosts, which may be particularly susceptible to pollutants and environmental stressors. Harbor estuaries are some of the most polluted environments on the planet, and due to rapidly fluctuating temperature and salinity a combination of environmental stressors may alter immune capabilities of resident fish, especially juvenile fish (Ludwig and Iannuzzi, 2005). To date, immunoassay approaches have been often used to understand the potential impacts of environmental stress on fish (Burnett, 1997; Grinwis et al., 2000; Rice, 2001; Rice and Arkoosh, 2002; Weeks and Warinner, 1986; Zelikoff J.T., 2000; Zelikoff, 1998), but virtually all of these studies have been carried out in adult fish. However, there have been few studies that adopt this approach with larval estuarine fish. In general it is thought that juvenile fish have limited immunocompetence (Zapata et al., 2006) and rely on innate immunity in early life (Rombout et al., 2005).

The mummichog, *Fundulus heteroclitus*, is distributed from Newfoundland to Florida (Bigelow and Schroeder, 1953) and is one of the most abundant species in high marsh habitats. This fish comprises a large percent of the total secondary productivity in high marsh habitats (Meredith and Lotrich, 1979). Also, mummichogs prefer the high marsh habitat and subsequently have high home range fidelity throughout their life cycle (Kneib, 1986). Due to the mummichog’s high
home range fidelity, this species is useful as an indicator species and for studying the effects of pollutants on estuarine fish (Frederick et al., (in press), Wassenberg and DiGiulio, 2004; Gaworecki et al., 2004; Hahn et al., 2004; Weis et al., 2003; Weis and Weis, 1982) However, little is known about the development and maturation of the immune system of juvenile mummichogs. The major aim of this study was to describe the disappearance of maternally-derived attributes of immunity and the appearance of autologous components of the immune system in mummichogs collected from a relatively pristine estuary, North Inlet Winyah Bay, SC USA. Such information will provide a model for testing the effects of various environmental stressors, including pollutants, on the developing immune system of estuarine fish.

This study demonstrates that egg IgM is quickly absorbed by the developing embryo, but juveniles did not produce IgM throughout the 5 week post-hatch (wph) observation period. In contrast, autologous lysozyme levels rose rapidly post-hatch and remain high throughout the 5-wph study. The expression of whole body Rag-1 was low until reaching peak levels at 3-wph, but rapidly fell to pre-hatching levels. The head kidney was the first organ to become lymphoid, followed by the spleen, and then the thymus. The thymus was not fully lymphoid until 3-wph. Overall, the developmental patterns of lymphoid tissues mirrored what has been previously described in other marine fish.

2.0 Materials and Methods

2.1 Animals

Adult mummichogs, Fundulus heteroclitus, were collected with baited minnow traps from North Inlet-Winyah Bay at Belle W. Baruch, Georgetown, SC USA. Eggs
and milt were collected manually according to previously published methods (Armstrong and Child, 1965) and fertilized eggs were transported to laboratories at Clemson University. After fertilization, developing mummichog embryos were maintained in petri dishes with enough artificial seawater (Instant Ocean) at 18 ppt to cover the eggs. Each day the embryos were observed and moribund animals were removed and water was exchanged. Upon hatching, larvae mummichogs were transferred to 1L aquaria in an aerated recirculating flow thru system that held 40-1L tanks. Water flowed out of the tanks into a biological filter bed, pumped thru a UV light, and finally into the tanks. Not all fry hatched on the same day, thus fish were held in separate tanks based on their hatch date. Newly hatched fry were maintained in well-aerated re-circulating aquaria for the duration of their development. In captivity, mummichog embryos typically hatch 12-14 days post-fertilization under a 12:14 light:dark photoperiod at 22°C. Fish were fed Artemia salina nauplii (Brine Shrimp Direct) until 4 weeks post-hatch (wph) and thereafter with commercial feed (Tetra).

Embryonic and larval mummichogs were sampled at the following stages of development: 1-, 2-, 3-, 4-, and 7-days post-fertilization (dpf), 1 day post-hatch (dph), 1-, 2-, 3-, 4-, and 5- weeks post-hatch (wph). For RNA and protein analysis, 3 replicates of pooled individuals of each age group were snap frozen in liquid nitrogen. For histological analysis, 3 individuals from 4- and 7-dpf, 1 dph, and 1-, 2-, 3-, 4-, and 5-wph were fixed in 4% paraformaldehyde in Hanks balanced salt solution (97g NaCl, 4.89g KCl, 12.2g glucose, .733g KH₂PO₄, 1.1g Na₂HPO₄·H₂O in 1L Di H₂O), hereafter referred to as HBSS. Samples for histology and protein/RNA were maintained at 4°C and -80°C respectively until further analysis.
2.2 Embryo and whole fish homogenates

Embryos and whole fish were homogenized in leukocyte lysis buffer (20 mM Tris HCL pH 8.0, 150 mM NaCl, 1 mM MgCl₂, 1 mM KCl, 50 mM EDTA, 1% NP-40 containing HALT (Promega) protease inhibitor cocktail). Also for each age group, 3 replicates of pooled individuals of each age group were homogenized. Protein concentrations were determined for all lysates with a bicinchoninic acid (BCA) assay (BioRad). Bovine serum albumin (BSA) was used as a standard protein and all samples were run in duplicate.

2.3 Enzyme linked immunosorbent assay (ELISA) for determination of total immunoglobulin M (IgM)

Immunoglobulin M (IgM) was detected in whole homogenates of embryos and larvae with a capture ELISA. Monoclonal antibody D58 (Rice and Xiang, 2000) was loaded onto 96 well plates (BD Falcon) at 100 µl per well as confluent hybridoma supernatant and incubated overnight at 4°C. The plate was then washed 3 times with 0.01 M phosphate buffered saline (PBS) containing 0.5% Tween 20 (PBS-Tw20). Fifty µg of total protein from the embryo and fish lysates were loaded onto the plates in duplicate and incubated for one hr. The plates were then washed with PBS-Tw20 and blocked for 1 hr with 3% BSA in PBS, followed by another three washes with PBS-Tw20. One hundred µl of mouse polyclonal antisera against mummichog IgM (diluted 1:1000 in PBS) were added to each well and the plate was incubated for 1 hr at room temperature. The plate was then washed three times with PBS-Tw20. One hundred µl of alkaline phosphatase (AP)-conjugated goat anti-
mouse Ig (H+L) antiserum (1:1000 in PBS) were added to all wells and the plate was incubated for 30 min. After three washes with PBS-Tw20 alkaline phosphatase activity was quantified by adding 100 µl of 1 mM p-nitrophenyl phosphate (Sigma) in alkaline phosphatase buffer 100 mM NaCl, 5 mM MgCl₂, 100 mM diethanolamine, pH 9.5), hereafter referred to as AP buffer. Plates were incubated for 30 min and the optical density for each well was recorded at 405 nm on a spectrophotometer (Bio-Tek Instruments, Inc.). The optical densities for each duplicate were averaged to obtain a data point for that sample. Data were then compared to one another as relative IgM levels.

2.4 Lysozyme activity

Whole embryo and fish lysozyme activity was determined using procedures described by (Parry et al., 1965) and modified after (Burton et al.). Twenty µl of sample were added in duplicate to wells of a 96-well microtiter plate and followed by the addition of 180 µl of 0.2 mg/ml heat killed Micrococcus lysodeikticus (Sigma) in 0.05 sodium phosphate buffer, pH 5.6. The reaction was carried out at room temperature (22°C) and read at 450 nm as soon as the M. lysodeikticus solution was added, then again every 10 min for a total of 40 min. One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 per minute.

2.5 SDS PAGE and Immunoblot for Lysozyme

All samples were diluted to the same protein concentration in lysis buffer, mixed with 4x sample buffer (40% glycerol, 240mM Tris-Cl pH 6.8, 8% SDS, .1% bromophenol blue with 5% (v/v) β-mercaptoethanol), and heat denatured by boiling
for 7 min. Samples were loaded onto 4-20% SDS-PAGE gels (BioRad) and subjected to electrophoresis. The separated proteins were then transferred to a PVDF membrane (Millipore) for immunoblotting. Proteins bound to PVDF were blocked for 1 hr with 3% BSA in PBS containing 0.1% gelatin, washed three times with PBS-Tw20, and probed for the relative expression of lysozyme protein using mAb M24-2 (Frederick et al., (in press); Marsh, 2007b). Following three washes with PBS-Tw20, the blots were incubated with AP-conjugated goat anti-mouse-IgG (diluted 1:1000 in PBS) for 30 min. The blots were then washed three times with PBS-Tw20 and alkaline phosphatase activity was visualized with 5-bromo-4-chloro-3-indoyl-phosphate/nitro-blue tetrazolium (NBT/BCIP) (Fisher Scientific) in AP buffer. Immunoblots were imaged and documented with a GS-710 densitometer (BioRad).

2.6 Isolation of total RNA and first strand synthesis from embryos and whole fish

For each age group, three groups of 15 individuals were pooled for RNA analysis. However, one group from 2-dpf had only 12 individuals. Total RNA from embryos and whole fish was isolated in TriReagent® at a concentration of 100 mg tissue/ml of TriReagent® per manufacturer’s protocol (Molecular Research Center, Inc.). Embryos were homogenized in a micro-bead homogenizer (Biospec Products, Inc.) with 2 mm zirconia beads while hatched fry were homogenized with a liquid nitrogen cooled mortar and pestle. Tissues were ground to a fine powder in liquid nitrogen and then added to TriReagent®. RNA purity was determined with a UV spectrophotometer (Eppendorf) at OD_{260/280} and a ratio > 1.7 was accepted for further reactions. To eliminate possible genomic DNA contamination, 1 µg of total
RNA was treated with amplification grade Dnase I (Invitrogen) prior to reverse transcription.

For expression analysis, total RNA (1 µg) was primed with oligo-(dT)12-18 and reverse-transcribed with SuperScript™ (Invitrogen), as described by the manufacturer. cDNA samples were stored at -20 °C until real time quantitative PCR analysis.

2.7 Real-time quantitative-polymerase chain reaction of Rag-1 expression during ontogeny

For real time quantitative-polymerase chain reaction of Rag-1, 100 ng of cDNA was added to 2x iQ SYBR Green supermix (BioRad) with 0.2 µM of forward and reverse primers, and water for a total volume of 15 µl. Quantitative PCR was run on an iCycler thermocycler (BioRad) with the following reaction conditions: initial denaturation at 95 °C, 40 cycles of denaturation at 95 °C for 30 s and annealing at 65 °C for 30 s, followed by a final extension at 55 °C for 1 min. Melt curve analysis was performed with continuous fluorescence from 65 to 95 °C at a temperature transition rate of 0.01 °C/s to determine amplification specificity. β-actin was used as the housekeeping gene and Rag-1 as the gene of interest.

Degenerative primers were originally produced for Rag-1 (Chapter 1), and after sequencing to confirm identity (DQ250438) new specific primers were produced for quantitative real-time PCR. The oligonucleotide primer sequences for each gene are listed below with their corresponding annealing temperatures in parentheses: β-actin forward ‘5′-TATGCAGAAGGAGATCACTGCC-3′ (56.3 °C), β-actin reverse ‘5′-ATCCACATCTGCTGGAAGGT-3′ (55.8 °C), Rag-1 forward ‘5′-GGAACTACGCCGAAGGT-3′ (56.7 °C), and Rag-1 reverse ‘5′-ACGCCAC
ACAGGTTTCATCT-3’ (56.7°C). Relative standard curves were constructed from a dilution series of plasmid cDNA (Chapter 1). The validity and suitability of using these cDNA plasmids for quantitative real-time PCR was also described in Chapter 1. To minimize variability due to reverse transcription efficiency and quality between samples, the data were normalized by dividing ng of Rag-1 by ng of β-actin. Quantitative results are reported as relative levels of Rag-1 normalized to β-actin. Each sample was run in quadruplicate, together with known dilutions of plasmid cDNA and appropriate non-template control.

2.8 Preparation of tissues for immunohistochemistry and light microscopy

Larvae and embryos were fixed with 4% paraformaldehyde in HBSS and held at 4°C until further processing. After fixation, the tissues were dehydrated through graded solutions of ethanol (ETOH) (25, 50, 75, 100, 100 %) for 30 min each at room temperature, cleared in xylene for 30 min at room temperature, and placed back in 100% ETOH for 30 min at room temperature. The samples were then infiltrated with Immunobed (Polysciences, Inc.) embedding media with graded solutions of ethanol and catalyzed resin (50 ETOH: 50 resin at 4°C overnight, 100% resin 6 hr at 4°C, and 100% resin overnight at 4°C) per manufacturer’s protocol. After overnight incubation in 100% catalyzed resin, the samples were embedded according to manufacturer’s specifications (0.4 ml of accelerator B/10 ml catalyzed resin). Sagital and oblique sections (1.5 μM) were cut on a microtome (Leica RM 2165), floated in a water bath, placed on colorfrost® slides (Fisher Scientific), and fixed to the slide on a hot plate.
All tissue sections were imaged on a Nikon E-600 light microscope with either a 4X, 10X, 20X, 40X, or 60X objective. Photomicrographs were taken using a Qimaging Micropublishing digital camera.

2.9 Immunohistochemistry (IHC) for lysozyme

For detection of mAb M24-2 positive cells, all sections were probed with the monoclonal antibody M24-2 (Frederick et al., (in press); Marsh, 2007b). Sections were etched with 100% ETOH for 2 min, hydrated with deionized water (DIH₂O) 3 times for 2 min each, washed with PBS 2 times for 5 min each, blocked for 15 min in blocking buffer (1% non-immune goat serum + 1% BSA). Tissue sections were then incubated for 1 hr with confluent hybridoma supernatant or in secondary antibody as negative control diluted 1:100 in blocking buffer, washed with blocking buffer 3 times for 5 min each, and incubated with gold-conjugated goat anti-mouse IgA+IgM+IgG (Ted Pella, Inc.) diluted 1:100 in blocking buffer. Tissues were washed in running DIH₂O for 5 min and still washed in DIH₂O for 5 min with constant dipping agitation. Finally, slides were silver enhanced with a silver enhancing kit (Ted Pella, Inc.) for 23 min, washed in running DIH₂O for 5 min, and stained with azure II/ basic fuchsin. Lastly, the sections were mounted and coverslipped prior to imaging.

2.10 Statistical analysis

Statistical differences of lysozyme enzyme activity between the age groups were tested with Analysis of Variance (ANOVA) followed by Neman Keul post hoc test (GraphPad). An \( \alpha \) value of \( p \leq 0.05 \) was considered to be statistically significant.
3.0 Results

3.1 Changes in whole embryo or whole tissue IgM in developing mummichogs

Immunoglobulin M (IgM) levels in whole embryo and larval homogenates were quantified using a capture ELISA. There was a three-fold reduction in IgM levels shortly after hatching, with the greatest decrease occurring between 1-dph and 1-wph, and levels remained low for the duration of the study (Fig. 2.1).

3.2 Lysozyme enzymatic activity and protein expression during mummichog development

Lysozyme enzymatic activity was detected using a standard assay, which quantifies the ability of lysozyme to lyse Micrococcus lysodeikticus. From 1-dph onward, lysozyme activity steadily increased until the end of the study period at 5-wph (Fig. 2.2). To determine if lysozyme activity was statistically different between age groups, an ANOVA was performed followed by a Neuman Keul’s post hoc test (Table 2.1). Activity levels were different between early (1-, 2-, 3-, 4-, 7- dpf, and 1- dph) and older fish (3- and 4- wph). In addition, lysozyme protein was analyzed by immunoblot using an antibody specific to mummichog lysozyme. Lysozyme protein was first detected at 7-dpf and decreased after hatching until 2-wph (Fig. 2.3). Thereafter, the amount of lysozyme protein gradually increased until it reached a maximum intensity at 5-wph.

3.3 Quantitative expression of Rag-1 during mummichog ontogeny

Expression of Rag-1 was quantified using real time PCR and normalized to the expression of the housekeeping gene β-actin. A melt curve analysis was performed for each quantitative real-time PCR run and a single specific melting peak
was observed indicating amplification specificity (data not shown). During mummichog development, \textit{Rag-1} expression increased at 2-wph and reached a maximum intensity at 3-wph. After 3-wph, \textit{Rag-1} expression dropped precipitously to pre-2 wph levels, where upon expression levels remained constant throughout the study period (Fig. 2.4). These data were reported as levels of \textit{Rag-1} expression relative to expression of \textit{\(\beta\)-actin}.

3.4 Appearance of lymphoid tissues in the head kidney

Clusters of renal tubules were first observed in the head kidney at 7-dpf prior to the appearance of hematopoietic tissues (Fig. 2.5 (a)). The head kidney was always found as a paired organ on either side of the head, anterior to the intestines, and dorsal to the yolk sac. At hatching (1-dph), there was a slight increase in renal tubules, and the first few hematopoietic cells appeared as scattered cells next to the renal tubules (Fig. 2.5 (b)). Morphologically, the hematopoietic cells appeared round, had dark basophilic staining, and some had a large nucleus to cytoplasm ratio. As fish aged, there was an increase in both the number of renal tubules and hematopoietic cells in the head kidney. From 2- to 4-wph, there was a transition in cell types of the head kidney from predominantly basophilic staining to acidophilic staining (Fig. 2.5 (d-f)). This shift in cell types occurred predominantly in cells that directly surrounded the renal tubules. However, by 5-wph acidophilic staining cells were found homogenously scattered throughout the head kidney parenchyma (Fig. 2.5(g)).
3.5 Appearance of lymphoid tissues in the spleen

The spleen was first observed at 1-wph located between the intestine and liver, closely associated with pancreatic tissue. As fish aged, the spleen became elongated, became less associated with pancreatic tissue, and increased in cellularity (Fig. 2.6 (a-c)). At 2- and 3- wph, a thin layer of pigmented mesentery surrounded a portion of the spleen (Fig. 2.6(b-c)). Pigmented mesentary was not observed at 4- or 5-wph (Fig. 2.6(d-e)). In all stages studied, the parenchyma appeared homogenous and was comprised of mostly erythrocytes, which appeared pink when stained, indicating a high amount of acidophilic residues. There were no definite divisions of white and red pulp, nor were melanomacrophage centers (MMC) observed.

3.6 Appearance of lymphoid tissues in the thymus

The thymus was closely associated with the pharyngeal epithelium and in close proximity to the head kidney. Thus, it was possible to observe the thymus while observing the head kidney. Thymic tissues appeared lymphoid at 3-wph in oblique and sagittal sections of juvenile mummichog (Fig. 2.7(a)). In all sections from 3-wph to 5-wph, the thymus appeared as a paired dorsal organ located posterior to the gill cavity in close proximity to the head kidney, and each organ wrapped around a muscle bundle (Fig. 2.7). The thymus and the head kidney were separated by sparse connective tissue and cartilage. At 3-wph, the thymic parenchyma consisted of a dense meshwork of small round basophilic staining cells. Due to their large nucleus to cytoplasm ratio the cells were probably immature thymocytes or lymphoblasts. From 3- to 5-wph, the thymus did not appear to change in size or
cellularity. A clear demarcation between the cortex and medulla could not be
distinguished in any of the sections.

3.7 Summary of lymphoid organ histogenesis during mummichog development

The appearance of lymphoid organs of three developing mummichogs, have been summarized in Table 2.2. The first appearance of lymphoid organs was as follows: renal tubules in the head kidney at 7-dpf, hematopoietic tissue in the head kidney at 1-dph, appearance of spleen at 1-wph, and the appearance of lymphocytes in the thymus 3-wph. It should be noted that whilst the appearance of tissues was noted, the tissues were not considered “lymphoid” until cells of the lymphoid lineage had infiltrated the tissue. For example, the head kidney structures had begun to appear as early as 7-dpf, but substantial lymphopoietic cells were not observed until 1-wph. In some individuals, identification of organs was not possible due to errors encountered in the embedding process.

3.8 Localization of mAb M24-2-positive cells during mummichog development

Tissue sections of mummichog were collected at different ages from embryo to larval stages and probed for lysozyme with the mAb M24-2. The appearance of lysozyme in different organs and tissues from three developing mummichogs, have been summarized in Table 2.3. Cells positive for M24-2 were first observed at 7-dpf and continued to increase in prevalence till 5-wph. At 7-dpf, a few M24-2 positive cells were observed in the brain, circulating thru blood vessels, and in the heart. At this stage, erythrocytes were the only M24-2 positive cells. By 1-dph, M24-2 positive cells appeared in the gills (Fig. 2.8(a)) and sparsely around renal tubules in the
parenchyma of the head kidney (Fig. 2.9 (a)). At 1-wph, M24-2 positive cells were observed in the liver, spleen (Fig. 2.10(a)), head kidney, gills, and in circulation. As fish aged, the number of M24-2 positive cells increased in these same tissues. In the head kidney, M24-2 positive cells were primarily seen surrounding renal tubules. Overall, erythrocytes were the predominant cell type to stain positive with M24-2.
Table 2.1. Newman-Keul’s post hoc test on lysozyme activity

<table>
<thead>
<tr>
<th></th>
<th>$p \leq 0.05$</th>
<th>$p \leq 0.001$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-dpf vs 2-dpf</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>1-dpf vs 3-dpf</td>
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<td>1-dpf vs 4-dpf</td>
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<tr>
<td>1-dpf vs 7-dpf</td>
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<tr>
<td>1-dpf vs 1-dph</td>
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<tr>
<td>1-dpf vs 1-wph</td>
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<td>1-dpf vs 2-wph</td>
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<td>1-dpf vs 3-wph</td>
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<td>1-dpf vs 4-wph</td>
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<tr>
<td>1-dpf vs 5-wph</td>
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</tbody>
</table>

Neuman Keul’s post hoc test performed after a one-way ANOVA. A $\alpha$ value of $p \leq 0.05$ was considered to be statistically significant. dpf= day post-fertilization, dph= day post-hatch, and wph= week post-hatch.
**Table 2.2**
Summary of lymphoid organ histogenesis during mummichog development

<table>
<thead>
<tr>
<th>Age</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Thymus</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 dpf</td>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>7 dpf</td>
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<td>---</td>
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</tr>
<tr>
<td>1 dph</td>
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<tr>
<td>3 wph</td>
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<tr>
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<td>+++</td>
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<td>*++</td>
</tr>
<tr>
<td>5 wph</td>
<td>+++</td>
<td><strong>+</strong></td>
<td>*++</td>
</tr>
</tbody>
</table>

Three individuals were examined per age group, dpf = days post-fertilization, dph = days post-hatch, wph = weeks post-hatch. + or – indicate presence or absence of organ in individuals. * indicate organ was not observed due to embedding error.
Table 2.3

The appearance of mAb M24-2 positive cells in different organs and tissues during mummichog development as detected by immunohistochemistry.

<table>
<thead>
<tr>
<th>Age</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Liver</th>
<th>Intestines</th>
<th>Blood Vessels</th>
<th>Gills</th>
<th>Brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 dpf</td>
<td>!</td>
<td>!</td>
<td>!</td>
<td>!</td>
<td>!</td>
<td>!</td>
<td>!</td>
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<tr>
<td>7 dpf</td>
<td>!</td>
<td>!</td>
<td>!</td>
<td>!</td>
<td>!</td>
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<td>!</td>
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<tr>
<td>1 dph</td>
<td>+</td>
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<td>!</td>
<td>!</td>
<td>+++</td>
<td>+</td>
<td>+</td>
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<tr>
<td>1 wph</td>
<td>+++</td>
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<td>!</td>
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<td>+++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3 wph</td>
<td>+++</td>
<td>+++</td>
<td>+++*</td>
<td>!</td>
<td>+++</td>
<td>+</td>
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<tr>
<td>4 wph</td>
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<tr>
<td>5 wph</td>
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<td>+</td>
<td>*</td>
<td>!</td>
<td>+++</td>
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<td>+</td>
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</table>

Three individuals were analyzed per age group, dpf = days post-fertilization, dph = days post-hatch, wph = weeks post-hatch. + or – indicate positive or negative staining of individuals (i.e. +--+ = one of three fish show positive staining). All sections were probed for lysozyme using mAb M24-2 and appropriate negative control. * indicate organ was not observed due to embedding error.
Fig. 2.1 Changes in whole embryo or whole tissue IgM during mummichog development at 1-, 2-, 3-, 4-, and 7-dpf; 1 dph; 1-, 2-, 3-, 4- and 5-wph as detected by ELISA. Number and bar of each point represent sample size and standard error of the means, respectively.
Fig. 2.2. Changes in the level of whole embryo or whole tissue lysozyme activity during mummichog development at 1-, 2-, 3-, 4-, and 7-dpf; 1 dph; 1-, 2-, 3-, 4- and 5-wph as determined by bioassay. Number and bar of each point represent sample size and standard error of the means, respectively.
Fig. 2.3. Changes in embryo or tissue lysozyme protein during mummichog development as determined by immunoblot. Mummichog lysozyme is approximately 14.5 kDa in size. From left, whole fish extract from mummichog at 1-, 2-, 3-, 4-, and 7-dpf; 1 dph; 1-, 2-, 3-, 4- and 5-wph.
**Fig. 2.4.** Quantitative expression of whole embryo or whole tissue $Rag-1$ expression from mummichogs, aged 4- and 7-days post-fertilization, 1-day post-hatch, and 1-, 2-, 3-, 4-, and 5-weeks post-hatch. Data were generated with the iCycler real-time PCR system.
Fig. 2.5. Appearance of lymphoid tissues in head kidney of the mummichog. Sagital and oblique sections of 7 dpf (a), 1 dph (b), 1-, 2-, 3-, 4- (c-f), and 5- wph (g) stained with azure II and basic fuchsin. Note, photomicrograph (g) includes two micrographs of 5-wph head kidney. Br: brain, I: intestine, and M: muscle. dpf=days post-fertilization, dph=days post-hatch, wph=weeks post-hatch. Black arrows indicate renal tubules.
Fig. 2.6. Appearance of lymphoid tissues in the spleen of the mummichog. Sagital and oblique sections of 1-, 2-, 3-, 4, and 5- wph (a-e) juvenile mummichog were stained with azure II and basic fuchsin. The spleen is adjacent to the liver and intestinal epithelium, closely associated with pancreatic tissue. wph= weeks post hatch, I= intestine, P=pancreatic tissue. Black arrows indicate the spleen.
Fig. 2.7. Appearance of lymphoid tissues in the thymus of the mummichog. Oblique sections (1.5 µM) of 3-wph (a), 4-wph (b), and 5-wph (c) mummichog. For each age, low magnification is on the left while high magnification is on the right. Thymic lobes are indicated by black arrows. wph = weeks post hatch, Hk: head kidney, M: muscle, Br: brain, I: intestine.
Fig. 2.8. Appearance of mAb M24-2 positive cells in the gills of developing mummichogs. Oblique sections (1.5 µM) of 1-5 wph (a-c) mummichog. Positive cells are indicated by black arrows. For all ages, positive stained sections (+) are on the left, while negative controls (-) are on the right. wph= weeks post-hatch.
Fig. 2.8. Appearance of mAb M24-2 positive cells in the gills of developing mummichogs. Oblique sections (1.5 µM) of 1-5 wph (a-e) mummichog. Positive cells are indicated by black arrows. For all ages, positive stained sections (+) are on the left, while negative (-) are on the right. wph= weeks post-hatch. (Continued)
**Fig. 2.9.** Appearance of mAb M24-2 positive cells in the head kidney of the developing mummichog. Oblique sections (1.5 µM) of 1-dph (a) and 1- to 5-wph (b and F) mummichog. For each age positive stained (appear black from silver stain) sections are on the left, while negative are on the right. Black arrows indicate positive cells. dph = day post-hatch and wph = weeks post-hatch.
Fig. 2.9. Appearance of mAb M24-2 positive cells in the head kidney of the developing mummichog. Oblique sections (1.5 µM) of 1-dph (a) and 1- to 5-wph (b and F) mummichog. For each age positive stained (appear black from silver stain) sections are on the left, while negative are on the right. Black arrows indicate positive cells. dph = day post-hatch and wph = weeks post-hatch. (Continued)
Fig. 2.10. Appearance of mAb M24-2 positive cells in the developing spleen of the mummichog. Oblique sections (1.5 μM) of 1- to 5-wph (a and c) mummichogs. For each age, positive stained sections (appear black from silver stain) are on the left (+), while negative controls are on the right (−). dph = day post-hatch and wph = weeks post-hatch.
Fig. 2.10. Appearance of mAb M24-2 positive cells in the developing spleen of the mummichog. Oblique sections (1.5 µM) of 1- to 5-wph (a and c) mummichogs. For each age, positive stained sections (appear black from silver stain) are on the left (+), while negative controls are on the right (-). dph= day post-hatch and wph= weeks post-hatch. (Continued)
4.0 Discussion

To determine if any attributes of immunity are passed on to the developing embryo from the female, mummichog IgM was quantified from fertilization onward. Interestingly, IgM was detected as early as 1-dpf in mummichog embryos. This suggests that the IgM levels in the egg were maternally derived. Maternally derived IgM has been documented in other fish species including, plaice (Bly et al., 1986), tilapia (Takemura, 1993), carp (Suzuki et al., 1994), sea bass (Breuil et al., 1997), rainbow trout (Castillo et al., 1993), red sea bream (Tanaka et al., 1999), and coho salmon (Yousif et al., 1995). However, among species, there is variability in the structure of egg IgM, egg distribution, and the amount deposited in the egg (Magnadottir et al., 2005). Hatching often coincides with a reduction in maternal IgM protein levels in post-hatch fish. However, neosynthesis of IgM occurs shortly after feeding (Breuil et al., 1997; Grontvedt and Espelid, 2003; Schroder et al., 1998; Iwama and Nakanishi, 1996; Magnadottir et al., 2005). In tilapia, an IgM like protein is detected in the pre-larval stages and drops off precipitously after hatching (Takemura, 1993). Takemura (1993) also noted that the production of autologous IgM by tilapia larvae occurred after yolk sac absorption and at the onset of feeding. In salmon, there is a gradual decline in egg Ig levels after fertilization until the onset of feeding at 130-dpf, and thereafter the levels increase rapidly (Olsen and Press, 1997). Thus, the transition from yolk sac larvae to the free feeding stage may result in a metabolic shift that stimulates the production of autologous IgM in some teleosts.

In this study, the onset of feeding did not coincide with IgM synthesis because mummichogs began feeding several days after hatching. More than likely, IgM production appears late in mummichog development, sometime after 5-wph.
Thus, juvenile mummichogs do not have aspects of humoral immunity at this time, and probably compensate with other forms of immune defense.

Late emergence of B cells and immunoglobulin is common in marine species compared to freshwater fish (Chantanachookhin et al., 1991; Magnadottir et al., 2005). In particular, mature B cells and IgM expression are not detected until 50-dph in sea bass (Breuil et al., 1997; dos Santos et al., 2000). In spotted wolfish, which hatches 4-5 months after fertilization, IgM positive cells appear between 1- and 4-weeks after hatching (Grontvedt and Espelid, 2003). In Atlantic cod, IgM positive cells are not detected until 8- to 10-wph (Schroder et al., 1998). In contrast, freshwater species such as rainbow trout and channel catfish, IgM positive cells appear as early as 1-wph (Castillo et al., 1993; Petrie-Hanson and Ainsworth, 2001). The precise role of maternal IgM in teleost eggs is unclear, though it may prevent the vertical transmission of pathogens, act as an opsonin for phagocytosis, activate complement pathways, or simply serve as a nutritional yolk protein (Magnadottir et al., 2005). It has yet to be determined what the exact role maternal IgM may have during mummichog development.

The activity of mummichog lysozyme on *M. lysodeikticus* gradually increased as the fish aged. In addition, lysozyme protein was first detected by immunoblot at 7-dpf, decreased after hatching, and then steadily increased from 3- to 5-wph. Using immunohistochemistry, lysozyme positive cells were found as early as 7-dpf circulating within blood vessels and sparsely scattered in brain tissue. By 5-wph, M24-2 positive cells were found distributed throughout the gills, spleen, brain, head kidney, intestines, heart, and blood vessels. Surprisingly, erythrocytes were the predominant cell type that stained positive for mAb M24-2 at all ages. To the
author’s knowledge, this has not been previously described in any other teleost. Though red blood cells were the predominant M24-2 positive cell type, a few other cell types, presumably phagocytes, were also observed to be positive for mAb M24-2. The distribution and localization of lysozyme in mummichog tissues is similar to what has been reported in plaice. In plaice, lysozyme positive cells were found in the spleen, kidney, leukocytes prepared from whole blood, cartilaginous support tissue of the gill filaments, gill lamellae, and between the muscle and the subcutis (Murray and Fletcher, 1976). However, in mummichogs, it appeared that the increase in lysozyme protein and activity coincided with the gradual increase in erythrocyte numbers, which is a completely novel observation; if indeed M24-2 is specific for lysoyzme.

To date, the only known source of lysozyme in fish is leukocytes, and neutrophils and macrophages in particular (Murray and Fletcher, 1976). Few studies have investigated the potential immunological role that fish red blood cells may have. In rainbow trout, acid-soluble erythrocyte extracts displayed antimicrobial activity against several bacterium (Fernandes and Smith, 2004). However, to the author’s best examination of the literature, there have been only two reports demonstrating immunohistochemical localization of lysozyme in fish tissues. In both reports, one on plaice (Murray and Fletcher, 1976) and the other in tilapia (Takemura, 1996), there was no demonstration of red blood cells staining positive for lysozyme. Nevertheless, the gradual increase in lysozyme and M24-2 positive cells is probably critical for larval mummichogs in which the appearance of adaptive immunity may not occur until after 5-wph. If further studies demonstrate that red blood cells are capable of producing lysozyme, then this will be a major discovery and will help elucidate an early aspect of innate immunity in developing fish. Lysozyme
production by red blood cells would protect the organism during the long period between hatching and complete formation of lymphoid organs leading to active acquired immunity.

Quantitative real-time PCR showed an elevation in Rag-1 expression at 2-wph, followed by an increase to a maximum intensity at 3-wph, after which expression declined to pre 2-wph levels for the duration of the study period. The observed peak in Rag-1 expression at 3-wph may be due to the appearance of putative lymphocytes in the thymus at 3-wph and/or the expansion of lymphopoietic cells in the head kidney. In fish, in situ hybridization has been the primary technique for analyzing tissue localization of Rag-1 expression. In zebrafish, Rag-1 expression is detected in the pancreas by 4-dpf (Danilova and Steiner, 2002), thymus at 4-dpf (Willett et al., 1997), and head kidney at 2-4 wpf (Lam et al., 2004). Lam et al. (2004) report that in zebrafish the rapid increase in Rag-1 expression levels between 1- and 3-wpf is contributed to an expansion in the thymocyte population as well as lymphocytes in the head kidney between 2- and 4- wpf. In carp, the appearance of the thymus coincided with a rapid increase in Rag-1 expression at 4-dpf and presumably the rearrangement of TCR (Huttenhuis et al., 2005). However, Huttenhuis et al. (2005) noted that the appearance of Rag-1 expression does not indicate the formation of functional T cells at that particular time. Yet, without documentation of Rag-1 expression by ISH, the source or location of Rag-1 expression in developing mummichogs is strictly speculative at this time. Perhaps future studies using antibodies against recombinant mummichog Rag-1 protein will allow IHC-localization in fixed tissues.
The kidney was the first organ of the immune system to contain putative cells of the lymphocyte lineage. Though renal tubules were present at 7-dpf, cells with hematopoietic morphological features were not observed in the head kidney until 1-wph. This is similar to what has been found in the gilthead seabream, where renal tubules appeared in 2 day old fish but hematopoietic cells were not present until 4 days later (Mulero et al., 2006). As the juvenile mummichog aged in this study, both the number of renal tubules and the proportion of hematopoietic tissue increased. This has also been reported in Atlantic cod (Schroder et al., 1998) and turbot (Padrós and Crespo, 1996). The mummichog spleen contained mainly erythrocytes and cells of the lymphocyte lineage were not observed in the spleen during the study period. Thus, the spleen at 5-wph was still relatively immature in appearance and lacked elements of maturity such as MMCs, which have been found in adult gulf killifish spleens (Marsh, 2007a), a close relative of the mummichog. Similarly, this has been reported in the flounder, Platichthys flesus, in which the spleen did not have mature attributes until the adult stage (Pulsford et al., 1994).

The thymus appeared lymphoid at 3-wph in developing mummichogs, and was located posterior to the gill cavity and continuous with the pharyngeal epithelium. Continuity between the thymus and the pharyngeal epithelium is characteristic of teleosts (Iwama and Nakanishi, 1996; Zapata et al., 2006; O'Neill, 1989; Padrós and Crespo, 1996; Liu et al., 2004). In this study, the cortex and medulla were not distinguishable, which is attributed to the lack of specific markers for thymocytes, due to age, or perhaps because corticomedullary regionalization does not occur in this teleost. In carp, the structure of the cortex and medulla were not well defined histologically until a specific marker was utilized with immunohistochemistry.
(Romano et al., 1999). From 3- to 5-wph, the mummichog thymus was mainly composed of basophilic staining cells, which had large nucleus to cytoplasm ratios. These cells were presumably lymphoblasts or immature thymocytes. Similar cell types have been described in the turbot at 15-25 dph (Padró and Crespo, 1996).

The thymus rapidly appeared lymphoid in mummichog larvae from 3- to 5-wph. Such developmental patterns and pace is similar to what has been described in marine teleosts (Table 2.4). The teleostean thymus typically undergoes thymic involution with age, although the details and progression of involution are not well understood (Iwama and Nakanishi, 1996; Zapata et al., 2006). In the present study, thymus involution was not observed at 5 wph, which is probably too early for involution to occur. Thus, the age at which thymus involution occurs in mummichog is yet determined.

In general, many marine species hatch with a relatively large yolk sac and under-developed organs (Falk-Petersen, 2005). Once hatched, mummichogs develop slowly and go thru several morphological changes before they are considered adults. Slow growth might not be critical for the mummichog, which is an opportunistic omnivore in an energy rich environment (Radtke and Dean, 1979). Yolk sac absorption occurs at stage 34 in mummichogs (Oppenheimer 1937), a period when many teleosts undergo a transition from the yolk sac stage to a free feeding larval stage. In larval mummichogs, this stage results in a decrease in feeding, which may indicate a shift in metabolism (Radtke and Dean, 1979). Such decreases in feeding may be one reason why mummichogs do not develop humoral immune capabilities at this stage. The relatively slow pace of organ development and maturation in mummichog has been also demonstrated in a number of other marine
teleosts whose larvae hatch at a less advanced stage of development and undergo a relatively long period of metamorphosis (Chantanachookhin et al., 1991).

There is a general agreement within the literature that the head kidney of marine fish develop prior to the thymus but that the thymus is the first to become lymphoid (Bowden et al., 2005; Chantanachookhin et al., 1991; Zapata et al., 2006). Needless to say, postulating defined developmental steps for lymphoid ontogeny in teleosts should be approached with caution. Reports in the literature are sometimes inconsistent when defining milestones in organ development. In particular, some report the appearance of small lymphocytes in the thymus as the first checkpoint (Solomom, 1978) while others consider the appearance of the alymphoid thymic analge as the first milestone (Boehm et al., 2003). Furthermore, discrepancy also occurs in the age that organs are reported to appear. Overall, it may be that sequence of development is more important than the exact age that an organ develops. Age may not be a good indicator of developmental status in fish due to differences in genetic plasticity, temperature, salinity, and early life history strategies.

In this study, cells of the lymphoid lineage were present in the head kidney prior to the appearance of lymphopoietic cells in the thymus, and the thymus was the last to appear lymphoid. Furthermore, the thymus was always found in close proximity to the head kidney, which may enable lymphoietic cells from the head kidney to “seed” the thymus. It has been hypothesized by others that hematopoietic cells from the head kidney colonize the rudiment thymus, possibly thru cell bridges between the thymus and kidney (Liu et al., 2004; Padrós and Crespo, 1996). However, it should be noted that this is purely speculative at this time and needs further attention. Overall, the presence of hematopoietic cells in the head kidney,
prior to the appearance of a “lymphoid” thymus, reiterates the notion that the head kidney is the primary hematopoietic organ in teleosts.

The mechanics of development are similar among teleosts, but there are differences with respect to the timing and sequence of developmental events. These differences may be attributed to variability in early life history strategy, genetic plasticity, or environmental factors. Thus, no one teleost can be a model for all species (Falk-Petersen, 2005). Unlike most higher vertebrates, many fish hatch at the embryonic stage of life and must undergo substantial changes before being considered adult. Furthermore, juvenile fish have little immunocompetence at hatching (Zapata et al., 2006), in that the lymphoid system is still developing and not all the structures and functions are present (Ellis, 1988), and most likely rely on innate immunity for immune defense (Rombout et al., 2005), which is likely the case for mummichogs. Though mummichogs are euryhaline and thermally-tolerant, they appeared to follow a similar pattern and pace to marine teleost lymphoid organ development. To the author’s knowledge, this is the first report that the lymphoid organs of high marsh estuarine fish develop in a similar sequence and pattern as strictly marine fish. Furthermore, this is the first study to suggest, though highly speculative, that developing red blood cells may be a source of lysozyme, which may provide an important barrier to aquatic pathogens during the period of lymphoid development and acquisition of acquired immunity.
Table 2.4. Sequence of lymphoid organ development in select marine teleosts

<table>
<thead>
<tr>
<th>Species</th>
<th>Age head kidney appears</th>
<th>Age spleen appears</th>
<th>Age thymus appears</th>
<th>First organ to be lymphoid</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Atlantic Cod <em>Gadus morhua</em> L.</td>
<td>5</td>
<td>4</td>
<td>28</td>
<td>thymus</td>
<td>(Schroder <em>et al.</em>, 1998)</td>
</tr>
<tr>
<td>Turbot <em>Scophthalmus maximus</em></td>
<td>1</td>
<td>10</td>
<td>18</td>
<td>thymus</td>
<td>(Padrós and Crespo, 1996)</td>
</tr>
<tr>
<td>Seabass <em>Dicentrarchus labrax</em></td>
<td>15</td>
<td>***</td>
<td>21</td>
<td>thymus</td>
<td>(Breuil <em>et al.</em>, 1997)</td>
</tr>
<tr>
<td>Antarctic teleost <em>Harpagifer antarcticus</em></td>
<td>1</td>
<td>28</td>
<td>28</td>
<td>N/A</td>
<td>(O’Neill, 1989)</td>
</tr>
<tr>
<td>Yellowtail <em>Seriola quinqueradiata</em></td>
<td>1</td>
<td>3</td>
<td>11</td>
<td>thymus</td>
<td>(Chantanachookhin <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td>Red sea bream <em>Pagrus major</em></td>
<td>0</td>
<td>3</td>
<td>11</td>
<td>thymus</td>
<td>(Chantanachookhin <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td>Japanese flounder <em>Paralichthys olivaceus</em></td>
<td>7</td>
<td>8</td>
<td>10</td>
<td>thymus</td>
<td>(Chantanachookhin <em>et al.</em>, 1991)</td>
</tr>
<tr>
<td>Flounder <em>Paralichthys olivaceus</em></td>
<td>1</td>
<td>8</td>
<td>15</td>
<td>head kidney</td>
<td>(Liu <em>et al.</em>, 2004)</td>
</tr>
</tbody>
</table>

Numbers: indicate days post-hatch (dph) and N/A: information not reported in the study cited.
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Frederick LA, van Veld PA, Rice CD. (in press). Bioindicators of immune function in creosote-adapted estuarine killifish, Fundulus heteroclitus. Journal of Toxicology and Environmental Health


Grinnis GC, Vethaak AD, Wester PW, Vos JG. 2000. Toxicology of environmental chemicals in the flounder (Platichthys flesus) with emphasis on the immune system: field, semi-field (mesocosm) and laboratory studies. Toxicol Lett 112-113:289-301.


The overall aim of this study was to describe the appearance of the immune system in the developing mummichog, *Fundulus heteroclitus*. The first research objective was to develop protocols for quantifying *Rag-1* expression, which offers the ability to follow expression in developing embryos and will lead to an understanding of lymphoid development in the mummichog. The goal for research objective II was to describe the disappearance of maternally-derived IgM and the appearance of various components of immunity, including lymphoid development, in mummichog. From objective I, the major outcomes were the cloning of mummichog *Rag-1*, expression of recombinant mummichog *Rag-1* protein, and finally the generation of a polyclonal antibody that recognized recombinant and cellular mummichog *Rag-1* protein. Moreover, *Rag-1* expression was analyzed in mummichog brain, intestine, heart, spleen, and head kidney. Expression of *Rag-1* was highest in the head kidney, which further validates this organ as a major site for lymphopoiesis in fish. For objective II, the major outcomes were that there is an overlap between maternal IgM and autologous production of lysozyme, which may help avoid a lapse in immune protection during early mummichog development (Fig.3.1). Furthermore, larval mummichogs probably rely on innate defense mechanisms until at least 5-wph. At 5-wph, mummichogs possessed all major lymphoid organs (head kidney, spleen, and thymus) but they did not have aspects of acquired immunity, at least in terms of tissue IgM. Needless to say, it is important to note that the appearance of lymphoid organs and cells does not indicate immunocompetence, and further study is needed.
to determine when immunocompetence is achieved in mummichogs. For example, mummichogs could be tested for immunocompetence via immunogen challenge. In general, previous studies on the development of lymphoid organs demonstrate that fish achieve immunocompetence after the appearance of lymphoid organs and after a critical number of mature cells have accumulated in the lymphoid organs.

Ultimately, these new reagents to mummichog Rag-1 described herein (antibodies, specific molecular probes) will enable investigators to examine expression in a variety of tissues and cell types, determine the intracellular location of the protein, and sites of expression that may be completely novel. In addition, the development of real-time PCR for quantifying Rag-1 expression and the availability of specific antibodies will allow environmental scientists to quantify expression of this key protein in fish exposed to environmental pollutants, or other stressors, known or suspected to be immunotoxic. Thus, Rag-1 expression may be a useful biomarker of immunocompetence in fish. Lastly, this study described lymphoid development in a teleost with a unique life history strategy, which contributes to the growing body of knowledge on comparative fish immunobiology.
Fig. 3.1 Major ontogenetic events during mummichog lymphoid development

- 1dpf
- 2dpf
- 3dpf
- 4dpf
- 7dpf
- 1dph
- 1wph
- 2wph
- 3wph
- 4wph
- 5wph

Hatching
- Appearance of hematopoietic tissue in head kidney
- Emergence of spleen

Feeding
- First peak in \textit{Rag-1} expression
- Highest peak in \textit{Rag-1} expression, appearance of organized lymphoid thymus

Maternal IgM

Autologous Lysozyme