Histologic Examination of Host-Parasite Interactions in the Gulf Killifish, Fundulus grandis

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HISTOLOGICAL EXAMINATION OF HOST-PARASITE INTERACTIONS
IN THE GULF KILLIFISH, Fundulus grandis

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Biological Sciences

by
Marlee Beckham Marsh
May 2007

Accepted by:
Dr. Charles. D. Rice, Committee Chair
Dr. Steven E. Ellis
Dr. Thomas E. Schwedler
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Dr. Alfred P. Wheeler
ABSTRACT

Little is known about the immunobiology of fish responses to parasitic infections. The aim of this study was to describe a model for the examination of fish-nematode interactions on the immunological front, and to develop new reagents for the study of this model. Gulf killifish, *Fundulus grandis*, were collected over two seasons from Simmon’s Bayou, Jackson County, MS, USA, pre Hurricane Katrina, and examined for endoparasites, particularly the larval nematode *Eustrongylides ignotus*. A baseline study was preformed to determine the prevalence, intensity, and histologic features of *E. ignotus* infection in *F. grandis*, and this study serves as the first description of its kind for this host-parasite model. In addition, two monoclonal antibodies, (mAb) M24-2 and 2C11, were characterized for their use in this project, as well as for general applications in fish immunobiology. Monoclonal antibody M24-2 was previously developed in this lab and found to recognize a ~ 14.5 kD protein in serum from all fish tested to date, and this antibody also recognized denatured hen egg lysozyme (HEL). Using mAb M24-2, cellular lysozyme protein was also found in lymphoid cell lysates, formalin-fixed and permeabilized lymphoid cells adhered to glass cover slips, and in plastic embedded head kidney and spleen tissue. In addition, mAb M24-2 was found to recognize most cells separated off percoll density gradients in a fraction known to contain fish phagocytes, primarily macrophages and neutrophils. Monoclonal antibody 2C11 was developed and determined to recognize a ~17 kD protein found in fish cell lysates, but in not plasma. Upon immune staining of glass adherent cells, it was determined that mAb 2C11 recognizes granule content of a small population of highly granulated cells, most likely eosinophilic granular cells (EGCs). Like mAb M24-2, mAb 2C11 was found to be cross
reactive in all species of fish tested; however, it is unable to bind its antigen in plastic embedded tissues.

Upon dissection of *F. grandis*, the larval nematode *E. ignotus* was found, as well as the apicomplexan *Calyptospora funduli* and metacercaria of a digenetic trematode. Histological descriptions of normal and infected head kidneys, spleen and livers from *F. grandis*, along with infected tissues probed with mAbs M24-2 and 2C11, were performed. There were no observable differences of cell types surrounding parasites in tissues, nor of immune staining, between infected and un-infected fish. There was no correlation between fish size, sex, parasite load and other parameters such as number of melanocyte macrophage centers in head kidney and spleen tissue, fatty liver and 2C11 positive cells.
DEDICATION

I dedicate this dissertation to my husband, Graham, for sticking by my side even when it meant we weren’t living in the same city. I could have never gotten through any of this without his love, patience, friendship and our continued support and respect for each other’s aspirations.

I further dedicate this dissertation to my parents, Buster and Kathy Beckham, who instilled in me the belief that I could climb any mountain in life and always reach the top. Without their unconditional love and unwavering support, none of this would be possible.
ACKNOWLEDGMENTS

I would first like to acknowledge my advisor, Dr. Charlie Rice, for flagging me down as a naive undergrad and welcoming me into his lab. Your encouragement, guidance, and confidence in me have molded me into the scientist I am today. Thank you for pushing me when I needed it, for your sense of humor, and for being so supportive of every aspect of my life. I could have never gotten this far without you.

I would also like to thank my committee members, Steve Ellis, Tom Schwedler, Tom Scott and Hap Wheeler. Thank you all, for encouraging me to become a PhD student in the first place, for teaching me invaluable skills in both the classroom and the lab, for good advice and honest feedback, and for the wonderful teaching experiences I have had at Clemson. Your direction has helped me grow as a scientist in so many ways.

I am also very grateful to my current and former lab mates, Alison Becker Springs, Lee Ann Frederick, Laura Hunt, Abby Babcock and Shannon Billings. I thank you for working extremely hard to help me with numerous studies over the past four and a half years and for making me laugh when I wanted to cry. I could not have done it without your help. Thank you also to the Tuesday night crew- Britt, Holly, Jon and Ant, for being amazing scientists, friends and an incredible support network. Your friendships have been invaluable to me.

Lastly, I would like to thank all of my family, friends and my husband, Graham, who understand that science doesn’t always stop for weekends or holidays. Your patience and encouragement have given me immeasurable strength and have made this journey that much easier.
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Adult nematodes of the Genus *Eustrongylides* parasitize the gastrointestinal tract of piscivorous birds (Measures, 1988; Spalding et. al, 1993). Infection of *Eustrongylides* spp. in piscivorous birds causes a sometimes-fatal disease known as Eustrongylidosis (Cole). *Eustrongylides* spp. eggs are shed into the water by infected birds and are subsequently consumed by aquatic oligochaetes, the first intermediate host, where they develop into second stage larvae (Lichtenfels and Stroup, 1985). A second intermediate host, usually a fish, then consumes the oligochaete worm. There are also numerous paratenic or aberrant hosts that are infected with larval *Eustrongylides* spp. (Lichtenfels, 1976; Lezama and Sarabia, 2002; Kuperman et al., 2004).

Gulf killfish, *Fundulus grandis*, are small estuarine fish that inhabit the shallow coastal marshes of the Gulf of Mexico from Florida to Cuba. The gulf killfish is one of the most abundant fish species found in high marsh habitats and has been used as a model for several immunological studies (Nevid and Meier, 1993; Nevid and Meier, 1994; Nevid and Meier, 1995; Roszell and Rice, 1998; Rice and Xiang, 2000). The Gulf killfish is a key food item for a variety of mammals, birds and fish in the Gulf of Mexico region, and is a popular bait fish for Gulf Coast fishermen (Solangi and Overstreet, 1980). The relative abundance of *F. grandis*, ease of culture, the growing body of literature specific to its immunobiology, and the prevalence of *E. ignotus* from *F. grandis* in this region support the use of these fish as good models for understanding host-nematode relationships in fish. To this end, *F. grandis* were collected on December 9, 2003 and June 28, 2004, and found to harbor *E. ignotus* in high numbers in the coelomic cavity on both occasions. Although the fact that *E. ignotus* infects *F.
Fundulus grandis has been previously reported (Overstreet, 2003), chapter one describes the prevalence of infection, as well as the histology of larval E. ignotus.

Chapter two describes the development and characterization of two monoclonal antibodies (mAb), M24-2 and 2C11, as these antibodies will be used to describe the fish-nematode model in chapter 3. Host-parasite immune responses of higher vertebrates are often mediated through Th$_2$ cells, resulting in the secretion of IL-5, eosinophilia, and immunoglobulin class-switching to an IgE (Sanderson, 1992; Pritchard et al., 1997; Behm, 2000; Prussin and Metcalfe, 2006). Host-parasite interactions in fish are one of the least understood areas of comparative immunology, mostly because immunoglobulin class-switching does not occur in fish, and evidence for Fc receptors on phagocytes is unequivocal at this time. However, the presence of eosinophilic granular cells (EGCs) in some species of fish suggests that there may be a role for these cells in the immune response to parasites (Cross and Matthews, 1993; Matsuyama and Iida, 1999; Kodama et. al, 2002; Sepulcre et. al, 2002; Dezfuli et al., 2002; Dezfuli et al., 2003; Dezfuli et al., 2004). Furthermore, any direct role(s) of neutrophils and macrophages in fish immune responses to parasites has yet to be determined. The underlying problem with advancing the knowledge of immunoparasitology in fish is a lack of suitable reagents to characterize cell types and general immune responses against parasites.

As described in Chapter 1, the gulf killifish, Fundulus grandis, is known to be a common host for the nematode parasite Eustrongylides ignotus, and thus holds promise as a reliable model for exploring basic aspects of host-helminth interactions. A closely related fish, the estuarine killifish, Fundulus heteroclitus, which is distributed along the east coast of North America, is also infected with Eustrongylides ignotus (von Brand, 1938; Cullinan, 1945; Weisberg, et al., 1986). Because of the similarities between F. grandis and F. heteroclitus,
immunological reagents developed in one typically cross-react in the other (Rice and Xiang, 2000; Van Veld, et al., 2005; Frederick, 2004). For example, monoclonal antibody M24-2 was generated against *F. heteroclitus* phagocytes and the specific protein appears to be lysozyme (Frederick, 2004). As described herein, mAb M24-2 recognizes lysozyme in both *F. grandis* and *F. heteroclitus*. The current study further characterizes this antibody and extends its possible uses in fish immunology.

Lysozyme is one of several humoral and cellular factors associated with innate immunity in all vertebrates. This enzyme (muramidase) breaks the β-1,4 glycosidic bond between *N*-acetylmuramic acid and *N*-acetylglucosamine in the cell wall of gram positive bacteria, and in association with complement components gram negative bacteria may be affected as well (Alexander and Ingram, 1992; Yano, 1996; Paulsen et al., 2001). In higher vertebrates, mature and fully differentiated macrophages are thought to be the primary source of lysozyme, as peripheral blood monocytes from humans and rodents produce less of the enzyme than do tissue macrophages and certain macrophage cell lines (Cross et al., 1988). In fish, lysozyme is expressed in cells of myeloid origin (Liu and Wen, 2002), and enzymatic activity can be found in mucus, serum, and eggs (Murray and Fletcher, 1976; Yousif et al., 1991; Yousif et al., 1994; Hanif et al., 2004). Moreover, there are seasonal variations in circulating lysozyme activity in some species of fish (Bowden et al., 2004).

Lysozyme production increases following proinflammatory stimuli. For example, β-glucan and lipopolysaccharide (LPS) increase lysozyme gene expression and enzyme activity in Atlantic salmon, *Salmo salar* L. anterior kidney macrophages (Paulsen et al., 2001). The kinetics of lysozyme gene expression and enzyme activity in this study were both time and dose dependent, with LPS being a stronger inducer. Immunization of adult female fish with potential bacterial pathogens leads to increased circulating lysozyme, which is passed to the
eggs after spawning (Yousif et al., 1991; Hanif et al., 2004). Furthermore, exposure to chemical contaminants, including mercury, selenium, copper, and co-planar PCBs modulate circulating lysozyme activity in fish (Low and Sin, 1998; Shariff, et al., 2001; Burton et al., 2002).

Quantifying lysozyme activity in a biological sample is routine and relatively simple to carry out (Parry et al., 1965; Ellis, 1990; Paulsen et al., 2001). Typically, a volume of fish serum, plasma, mucus, or egg yolk is mixed with a suspension of heat killed *Mycococcus lysodieticus* at an acidic pH. The optical density of the solution is then measured in the beginning and at a later time point for spectrophotometric analysis, or 18 – 24 hr later for agar-based systems that quantify a zone of lysis. The rate of decrease in optical density or diameter of the zone of lysis is compared to a known lysozyme standard such as hen egg lysozyme (HEL), and enzymatic activity is calculated as units of HEL activity (Parry et al., 1965) or HEL equivalents (Karrow et al., 1999).

The primary draw back for measuring lysozyme activity is that a minimum of 20 - 40 µl of sample is required for microtiter assays, a volume not readily available from small fish commonly used in laboratory studies. Moreover, the significance of circulating enzyme activity can be questioned. Plasma or serum lysozyme is bathed in a medium of slightly alkaline pH, which is far above the optimal pH of 5.9 for enzymatic activity. An acidic pH is more typical of the intracellular lysosomal compartment. Based on this difference between the optimal assay pH for circulating lysozyme activity and the actual pH of the serum or plasma sample, one can question whether or not measuring circulating enzyme activity is biologically significant.

Nonetheless, measuring lysozyme activity in fish from small quantities of sample can be problematic. Therefore, an alternative means for quantifying circulating lysozyme is
needed. The development of a lysozyme-specific antibody that cross reacts with a variety of fish species would allow researchers to quantify this important marker of innate immune responses in very small amounts of material.

In addition to characterizing mAb M24-2, this study describes the development and characterization of mAb 2C11, which recognizes a protein found only in highly granulated cells that may be equivalent to eosinophilic granular cells (EGCs) found in various species of fish (Ainsworth, 1992). Since EGCs may function like eosinophils of higher animals (Roszell and Rice, 1998), this antibody reagent may be useful in identifying some of the roles of EGCs in fish-parasite interactions in not only *F. grandis*, but other species of fish as well.

Most studies on fish EGCs have focused on tissues such as intestine, gills and skin (Ellis, 1985; Vallejo and Ellis, 1989; Powell et al., 1991; Holland and Rowley, 1998; Reite, 1998). These tissues are not traditionally considered primary or secondary immune organs of teleost (Zapata et al., 1996), leaving a void in what is known about EGC distribution in lymphoid tissues such as head kidney and spleen. Furthermore, initial studies of EGCs have demonstrated their involvement in parasite responses (Flano et al., 1996; Reite, 1998; Matsuyama and Iida, 1999; Dezfuli et al., 2000; Dezfuli et al., 2004), making these cells a logical focal point in understanding fish immunoparasitology.

Fish innate immune responses are routinely evaluated as indicators of immune function and status following exposure to pathogens, biological response modifiers, immunotoxicants, and nutritional regimes (Zelikoff et al., 2000; Bols et al., 2001; Rice, 2001; Rice and Arkoosh, 2002). The work in chapter two describes the development and characterization of mAbs for detecting, localizing, and quantifying cellular immune responses in fish as a possible indicator of immune status.
In chapter three, the host-parasite model of *F. grandis* and endoparasites found to infect the fish is examined. Despite the large body of literature demonstrating that fish harbor a wide variety of both ectoparasites and endoparasites (Ribelin and Migaki, 1975; Zapata et al, 1996) very little is known about fish immune responses following infection. Evidence suggests that both innate and adaptive immune responses are involved in host-parasite relationships in all vertebrates (Jones, 2001). Nevertheless, many aspects of immunity to parasites in fish have yet to be described.

There are several well-established models for examining the immune responses of fishes to parasites. Ectoparasite models include the monogeneans *Gyrudactylus* spp. and *Dactylogyrus* spp. and the copepod *Lepeoptheirus salmonis*, which have been studied almost exclusively in salmonids (Jorgensen et al., 2007). Haematozoic parasite models consist of the Kinetoplastids’ *Cryptobia* spp. and *Trypanosoma* spp and their infectivity of trout, salmon and carp (Ardelli and Woo, 1998). However, detailed knowledge of histiozoic and coelozoic parasites of fish is lacking. The best-known models of histiozoic and coelozoic parasites of fish are *Myxobolus cerebralis*, the source of whirling disease in salmonids, and *Ceratomyxa shasta*, another myxosporean infecting salmonids (Jones, 2001). The effects of another histiozoic parasite, the digenean trematode *Diplostomum spathaceum*, have been studied immunologically in rainbow trout. There are no well-characterized nematode models for fish immunoparasitology, and most of the fish models are salmonids. It is therefore important to further the current understanding of immune responses of fish to parasites by expanding not only the types of parasite studied, but the type of fish mounting the response as well.

As mentioned, Gulf killifish are small estuarine fish that inhabit the shallow coastal marshes of the Gulf of Mexico from Florida to Texas, and is one of the most abundant fish species found in high marsh habitats (Solangi and Overstreet, 1980). Several studies
demonstrate the utility of *F. grandis* as a model for fish immunology and immunotoxicology (Nevid and Meier, 1993; Nevid and Meier, 1994; Nevid and Meier, 1995; Roszell and Rice, 1998; Rice and Xiang, 2000) and the observation that *F. grandis* are heavily colonized by endoparasites (Chapter 1) support the use of *F. grandis* as a good model for furthering out understanding of host-parasite relationships in fish.

*Eustrongylides ignotus* is a histiozoic nematode whose definitive hosts include piscivorous Gioniiformes such as herons and egrets. *E. ignotus* infection causes large scale die-offs in nesting Gioniiformes. Adult birds shed nematode eggs into the water in feces and the eggs are in turn consumed by an aquatic oligochaete, the first intermediate host. Numerous fish species can acquire *E. ignotus* infection by ingesting an infected oligochaete, thus becoming a second intermediate host to the nematode (Coyner et al., 2003).

*Calyptospora funduli* is an apicomplexan parasite that infects numerous species of Cyprinodontidae fishes included *Fundulus grandis, F. heteroclitus, F. similis, F. pulvereus, F. jenkinsi, F. confluentus* and the Atherinidae *Menidia beryllina* (Fournie and Overstreet, 1993). Although *Calyptospora funduli* infection of *F. grandis* has been well characterized (Fournie et al., 2000), host-parasite interactions of apicomplexan infection of fish in general have been poorly described (Jones, 2001).

In the study described in chapter three, a population of *F. grandis* from Simmon’s Bayou, Jackson County MS was examined over a two year period. Fish were necropsied and examined for presence of parasites, and their head kidney, spleen and livers were prepared for histological examination. Larvae of *E. ignotus* were found in the coelom, and after histologic sectioning *C. funduli* and *E. ignotus* were detected in liver tissue, and an unidentifiable digenetic trematode metacercaria was observed in the head kidney and liver. Tissues were examined for their architecture as well as the abundance and degree of
infiltration of phagocytes using monoclonal antibodies 2C11 and M24-2, which recognize fish granulocyte protein and fish lysozyme, respectively (Chapter 2).


FIRST DESCRIPTION OF THE LARVAL NEMATODE
Eustrongylides ignotus PREVALENCE, INTENSITY,
AND HISTOLOGY FROM THE GULF
KILLIFISH, Fundulus grandis, GULF OF
MEXICO, USA

Abstract

The nematode parasite Eustrongylides ignotus is known to infect fish species throughout the Gulf of Mexico and the Eastern coast of the United States. This report is the first to describe the infection of E. ignotus in the second intermediate host Fundulus grandis, the Gulf killifish. Fish were collected over two years and examined for presence of larval worms. Worms were found encysted or not in the coelom of adult killifish. Several were found encysted in the liver. The average prevalence for both collections was 63% with the mean intensity 3.3. Larval worms were also processed for histological examination and tissues described.
Introduction

Adult nematodes of the Genus *Eustrongylides* parasitize the gastrointestinal tract of piscivorous birds (Measures, 1988; Spalding et. al, 1993). Infection of *Eustrongylides* spp. in piscivorous birds causes a sometimes-fatal disease known as Eustrongylidosis (Cole).

*Eustrongylides* spp. eggs are shed into the water by infected birds and are subsequently consumed by aquatic oligochaetes, the first intermediate host, where they develop into second stage larvae (Lichtenfels and Stroup, 1985). A second intermediate host, usually a fish, then consumes the oligochaete worm. There are also numerous paratenic or abberant hosts that are infected with larval *Eustrongylides* spp. (Lichtenfels, 1976; Lezama and Sarabia, 2002; Kuperman et al., 2004).

Gulf killifish, *Fundulus grandis*, are small estuarine fish that inhabit the shallow coastal marshes of the Gulf of Mexico from Florida to Cuba. The gulf killifish is one of the most abundant fish species found in high marsh habitats and has been used as a model for several immunological studies (Nevid and Meier, 1993; Nevid and Meier, 1994; Nevid and Meier, 1995; Roszell and Rice, 1998; Rice and Xiang, 2000). The Gulf killifish is a key food item for a variety of mammals, birds and fish in the Gulf of Mexico region, and is a popular bait fish for Gulf Coast fishermen (Solangi and Overstreet, 1980). The relative abundance of *F. grandis*, ease of culture, the growing body of literature specific to its immunobiology, and the prevalence of *E. ignotus* from *F. grandis* in this region support the use of these fish as good models for understanding host-nematode relationships in fish. To this end, *F. grandis* were collected on December 9, 2003 and June 28, 2004, and found to harbor *E. ignotus* in high numbers in the coelomic cavity on both occasions. Although the fact that *E. ignotus* infects *F. grandis*...
has been previously reported (Overstreet, 2003), we herein describe the prevalence of infection, as well as the histology of larval *E. ignotus*.

**Materials and Methods**

Adult Gulf Killifish, *Fundulus grandis*, were collected from Simmon's Bayou, Jackson County, MS USA using baited minnow traps and shipped overnight in an oxygen-saturated container to laboratory housing facilities at Clemson University. Fish were transferred to 100 L tanks containing aerated 12 parts per thousand artificial sea water (Instant Ocean) and maintained at 27 °C. After five days acclimation, *F. grandis* were anesthetized with 1 g/L tricaine methane sulfonate (MS-222) in ambient artificial seawater, weighed, measured, sexed and observed for any obvious external signs of infection. Fish were then bled and decapitated. Subsequently, fish were eviscerated and examined for the presence of larval *Eustrongylides ignotus*. The number and location of worms were recorded, as well as the general appearance of the coelom. If larval *E. ignotus* were found upon dissection, their location was noted and whether the larvae were encysted.

**Tissue collection**

In addition to the collection of larval worms, fish head kidneys and spleen were collected during this study and were placed in neutral buffered formalin (NBF) or snap frozen in O.C.T. media. Since larval worms are almost always coiled in "knots", some of the worms were placed in fixative as entire knots, while others were cut with a single-edged razor blade into smaller pieces. Worms were fixed in NBF and dehydrated using a graded progression of alcohol, cleared with Xylenes, and returned to 100% EtOH to await embedding. Tissues were then pre-infiltrated with a 50:50 solution of Immunobed
((Polysciences, Warminster, PA) overnight, infiltrated in 100% Immunobed overnight, and embedded in size appropriate molds. Blocks were desiccated for at least 4 days before microtomy. All tissues were sectioned at 2µm and stained with 0.5% azure II and 0.25% basic fuchsin in a 0.5% sodium-borate solution, mounted with Cytoseal XYL (Richard-Allan Scientific), and imaged. Micrographs were taken using a Q imaging Micropublisher camera mounted onto a Nikon Eclipse E600 microscope.

Results and Discussion

Some reports suggest that *Eustrongylides* spp. infection in fish is indicated by a "droopy" belly, or granulomatous lesions (Cullinan, 1945; Cooper et al., 1978; Crites, 1982; Coyner et al., 2002; Coyner et al., 2003). However, in this study not all fish presented with such symptoms. Some females presented with droopy bellies, but these females were often gravid, not infected (Figure 1.1). All larvae collected were pink to red, encapsulated or not, but all were tightly coiled as previously described by (Coyner et al., 2002). Encystment was typically observed in the mesenteries, although there were three cases where larval *E. ignotus* were found encysted in the liver. Larval worms were not always encysted. Most larvae were located in the coelom within the mesenteries and some were found encysted within the liver. No other signs of infection or inflammation were noted.

The prevalence and intensity of infection was found to be high (Table 1.1). Prevalence is the number of fish infected divided by the total number of fish sampled. Intensity is the total number of worms found per fish. Over half of the 25 fish collected in December were infected with larval *E. ignotus*. The highest number of worms recorded from a single fish was from a female of 11.5 cm in length containing 17 larval worms. The average
intensity was 4.7 during this collection period. The *F. grandis* collected late June demonstrated even higher prevalence levels at 68%. While prevalence was higher during this collection time, intensity was lower with an average of 2.7 larvae per infected fish. The highest incidence of infection coming from a single fish was 8 larvae in female of 5 cm in length.

The cuticle of larval *E. ignotus* is extremely basophilic (Figure 1.2). The cuticular ridges, or anulations, associated with *Eustrongylides* spp. and most other species of nematodes (Roberts and Janovy, 2000), are prominent. The hypodermis below the cuticle is difficult to distinguish. Beneath the hypodermis longitudinal arrangements of somatic musculature are distributed along the entire length of the worm. In nematodes, the musculature is composed of a contractile portion which takes up basic stains and a noncontractile cell body called a myocyton (Figure 1.2.C). Specifically, in *E. ignotus*, muscular arrangement is coelomyarian, with the contractile portion extending medially into the coelom (Lee, 2002).

The intestine of nematodes is a simple tube that extends from the esophagus to the proctodeum, the end portion of the alimentary canal (Roberts and Janovy, 2000). It is a nonmuscular structure composed of tall, simple columnar epithelium with a prominent brush border of microvilli (Figure 1.2.B). Intestinal cells sit on a basement membrane that attach to the body wall by random extensions of musculature (Roberts and Janovy, 2000). Although the basement membrane of the intestinal cells is clearly observable in *E. ignotus*, muscular extensions were not observed.

Nematodes of the order Dioctophymatida have highly developed esophageal regions as seen in Figure 1.2 (Lee, 2002). The muscular esophagus is extremely basophilic and a faint lumen is visible curving down to the intestine.
A female larval *E. ignotus* is characterized in Figure 1.4. Oviduct runs along side the intestine in the posterior portion of the worm (Figure 1.4.A, B and C). Oviduct is lined by simple columnar epithelium and its lumen is known as the axial rachis (Leake, 1975).

As mentioned, some of the larval worms were found to be encapsulated (Figure 1.5) in the coelom or on the liver. Figure 1.6 illustrates high power images of an encapsulated worm. At various locations on the encysted worm, we observed clots of fish blood cells (Figure 1.6. A). It is unclear if these cells are an artifact from the dissection and fixation procedures or if they serve some sort of immunological purpose for the fish. The connective tissue capsule (Figures 1.6.B, 1.6.C, 1.6.D) is composed of fibroblasts, collagen fibers and has blood vessels running throughout. This particular cyst was found embedded in the liver (1.6.B).

Observations of *E. ignotus* infection in *F. grandis* are similar to those found for most *Eustrongylides* spp in other fish. As reported by others (Weise et al., 1977; Coyner et al., 2002; Coyner et al., 2003), larval *Eustrongylides* in fish hosts were pink to bright red in color, tightly coiled, and some were found encapsulated in the coelom.

However, some aspects of *E. ignotus* infection in *F. grandis* were different from other studies. Firstly, Coyner et al. (2002) reported the highest prevalence of infection occurring in the winter months (December-March), with the summer months (June-September) falling in between spring (March-June) and fall (September- December). In this study, the highest prevalence was in the summer (68% compared to 53% in the winter). Furthermore, several studies report most larvae were found to be encapsulated, but we did not find this to be the case in *F. grandis* (von Brand, 1937; von Brand, 1938; von Brand and Cullinan, 1943; von Brand and Simpson, 1944; Coyner, 2002). Rather, the majority of larvae were free in coelomic cavity.
Coyner et al. (2002) reported that in mosquitofish, smaller fish were infected more often than larger species and that there was no prevalence difference between sexes. However, many others report that larger fish have a greater chance of infection than do smaller fish, and as fish increase in size and age, the more larvae they are likely to acquire (Measures, 1988; Cullinan, 1945; Crites, 1982). We found no correlation between size of the fish and number or larval worms (data not shown—see Ch. 3).

This is the first report of *E. ignotus* histology. Most of the connective tissue capsules from encysted larval worms were removed for fixation so that the fixative would better penetrate the worm. However, even with the capsules removed, it is still difficult for fixative and embedding media to completely penetrate the tissue of larval worms. Upon microtomy, un-infiltrated worm sections were distinguished by having a darker color than the white to clear infiltrated portions. Also upon cutting, the un-infiltrated portions would pop out of the plastic blocks. To remedy this in the future, it is recommended that investigators repeatedly pierce the cuticle with a fine gauge needle before fixation, thus allowing more complete penetration of tissue.

Historically, nematode histology has centered on cross-sections of *Ascaris lumbricoides* or *A. suum*, the giant intestinal roundworm of humans and pigs, respectively (Leake, 1975). While cross-sections of *Ascaris* spp. show many of the organs found within the organism, it is more difficult to orient worms in plastic embedding media at that angle. Sagittal sections are the easiest to embed and cut, and these sections show larger portions of the tissues present, which is advantageous when conducting any type of immune staining.

This study provides baseline data for future use of this fish-nematode model to be used in immunological studies.
Acknowledgments: We would like to thank Dr. Robin Overstreet for collection and shipment of *F. grandis* as well as identification of the larval *Eustrongylides*. 
Figure 1.1. Digital photos of healthy looking A) *Fundulus grandis* female. B) Coelomic contents of A. C) Eight larval *Eustrongylides ignotus* dissected from B.
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Table 1.1 Prevalence and intensity of *E. ignotus* from *F. grandis* as recorded from two different collection dates. Both collections from Simmon's Bayou in Jackson Count County, Mississippi, USA.
Figure 1.2. (A) Larval *Eustrongylides ignotus* longitudinal section through the intestinal region. 40x. (B) Higher power view of intestine characterized by simple columnar epithelium with microvilli resting on a basement membrane (arrowhead). 200x. (C) Body wall of larval worm consisting of outer cuticle (white bar) with hypodermis, underlying muscle composed of the contractile portion (gray bar) and noncontractile myocyton (arrow). 200x.
Figure 1.3. Head regions of *E. ignotus* at different magnifications. (A) Low power image of anterior portion of larval worm. 20x. (B) Inset of A at higher power. 100x. (C) Inset of B at higher power. Esophageal region. 200x
Figure 1.4. Gonads of larval *E. ignotus*. (A) Low power image of posterior end of worm with intestine (i), gonads (g) and body wall in view. 20x (B) Inset of A at higher power. 100x (C) Inset of B at higher power. Oviduct 400x.
Figure 1.5. Encapsulated larval *E. ignotus*. Photomontage of 20x images reconstructing a larval *E. ignotus* encysted on the liver (arrowheads).
Figure 1.6. High magnification images of an encapsulated *E. ignotus*. (A) Section through intestinal region of worm with fish blood clot attached to cuticle (arrow). 100x. (B) Portion of *F. grandis* liver (L) attached to cyst by thin layer of connective tissue (arrow). 100x. (C) View of connective tissue capsule encysting larval *E. ignotus*. 200x. (D) Connective tissue capsule with vascular source (arrow). 200x.
Literature Cited


Abstract

In this study, are reported the development and characterization of two mAbs, M24-2 and 2C11, which specifically recognize lysozyme and a granulocyte protein of eosinophilic granular cells (EGCs), respectively. Monoclonal antibody M24-2 is useful for detecting and quantifying lysozyme, and was developed from mummichog, Fundulus heteroclitus, leukocytes and tested for cross-reactivity in different species of teleosts, and compared to hen egg lysozyme (HEL). Quantification of lysozyme has historically been accomplished by measuring its enzyme activity against killed Micrococcus lยสodieticus using whole serum or plasma at a low pH, however the significance of lysozyme activity in serum or plasma is questionable because the pH of body fluids is normally above 7, far above the optimal pH for lysozyme activity. Furthermore, the amount of serum or plasma required for standard lysozyme activity exceeds what can easily be acquired from small fish, thus making lysozyme a difficult endpoint to measure in limited sample volumes. A single protein of ≈ 14.5 kD mass was identified by the mAb in all serum or plasma samples, as well as cell lysates sample from all fish tested to date, and this antibody recognized denatured HEL. Using mAb M24-2, cellular lysozyme protein was detected in both formalin-fixed and permeablized lymphoid cells adhered to glass cover slips and in plastic embedded tissue sections. The cellular sources of lysozyme in fish are macrophages and neutrophils and these phagocytes were separated by density centrifugation using Percoll gradients, obtained from the 1.060/1.080 region and M24-2 recognized more cells in this region than any other fraction. Monoclonal
antibody 2C11 recognizes an intracellular protein of \( \approx 17kD \) mass, but does not detect this protein in plasma. Utilizing mAb 2C11, an intracellular protein in highly granulated cells was identified in cells adherent to glass cover slips and in spleen cryosections, but not in plastic embedded tissue sections.
Introduction

Host-parasite immune responses of higher vertebrates are often mediated through \( \text{Th}_2 \) cells, resulting in the secretion of IL-5, eosinophilia, and immunoglobulin class-switching to an IgE (Sanderson, 1992; Pritchard et al., 1997; Behm, 2000; Prussin and Metcalfe, 2006). Host-parasite interactions in fish are one of the least understood areas of comparative immunology, mostly because immunoglobulin class-switching does not occur in fish, and evidence for Fc receptors on phagocytes is unequivocal at this time. However, the presence of eosinophilic granular cells (EGCs) in some species of fish suggests that there may be a role for these cells in the immune response to parasites (Cross and Matthews, 1993; Matsuyama and Iida, 1999; Kodama et. al, 2002; Sepulcre et. al, 2002; Dezfuli et al., 2002; Dezfuli et al., 2003; Dezfuli et al., 2004). Furthermore, any direct role(s) of neutrophils and macrophages in fish immune responses to parasites has yet to be determined. The underlying problem with advancing the knowledge of immunoparasitology in fish is a lack of suitable reagents to characterize cell types and general immune responses against parasites.

As described in Chapter 1, the gulf killifish, *Fundulus grandis*, is known to be a common host for the nematode parasite *Eustrongylides ignotus*, and thus holds promise as a reliable model for exploring basic aspects of host-helminth interactions. A closely related fish, the estuarine killifish, *Fundulus heteroclitus*, which is distributed along the east coast of North America, is also infected with *Eustrongylides ignotus* (von Brand, 1938; Cullinan, 1945; Weisberg, et al., 1986). Because of the similarities between *F. grandis* and *F. heteroclitus*, immunological reagents developed in one typically cross-react in the other (Rice and Xiang, 2000; Van Veld, et al., 2005; Frederick, 2004). For example, monoclonal antibody M24-2 was generated against *F. heteroclitus* phagocytes and the specific protein appears to be
lysozyme (Frederick, 2004). As described herein, mAb M24-2 recognizes lysozyme in both *F. grandis* and *F. heteroclitus*. The current study further characterizes this antibody and extends its possible uses in fish immunology.

Lysozyme is one of several humoral and cellular factors associated with innate immunity in all vertebrates. This enzyme (muramidase) breaks the $\beta$-1,4 glycosidic bond between N-acetylmuramic acid and N-acetylglucosamine in the cell wall of gram positive bacteria, and in association with complement components gram negative bacteria may be affected as well (Alexander and Ingram, 1992; Yano, 1996; Paulsen et al., 2001). In higher vertebrates, mature and fully differentiated macrophages are thought to be the primary source of lysozyme, as peripheral blood monocytes from humans and rodents produce less of the enzyme than do tissue macrophages and certain macrophage cell lines (Cross et al., 1988). In fish, lysozyme is expressed in cells of myeloid origin (Liu and Wen, 2002), and enzymatic activity can be found in mucus, serum, and eggs (Murray and Fletcher, 1976; Yousif et al., 1991; Yousif et al., 1994; Hanif et al., 2004). Moreover, there are seasonal variations in circulating lysozyme activity in some species of fish (Bowden et al., 2004).

Lysozyme production increases following proinflammatory stimuli. For example, $\beta$-glucan and lipopolysaccharide (LPS) increase lysozyme gene expression and enzyme activity in Atlantic salmon, *Salmo salar* L. anterior kidney macrophages (Paulsen et al., 2001). The kinetics of lysozyme gene expression and enzyme activity in this study were both time and dose dependent, with LPS being a stronger inducer. Immunization of adult female fish with potential bacterial pathogens leads to increased circulating lysozyme, which is passed to the eggs after spawning (Yousif et al., 1991; Hanif et al., 2004). Furthermore, exposure to chemical contaminants, including mercury, selenium, copper, and co-planar PCBs modulate
cycling lysozyme activity in fish (Low and Sin, 1998; Shariff, et al., 2001; Burton et al., 2002).

Quantifying lysozyme activity in a biological sample is routine and relatively simple to carry out (Parry et al., 1965; Ellis, 1990; Paulsen et al., 2001). Typically, a volume of fish serum, plasma, mucus, or egg yolk is mixed with a suspension of heat killed *Mycococcus lysodeiticus* at an acidic pH. The optical density of the solution is then measured in the beginning and at a later time point for spectrophotometric analysis, or 18 – 24 hr later for agar-based systems that quantify a zone of lysis. The rate of decrease in optical density or diameter of the zone of lysis is compared to a known lysozyme standard such as hen egg lysozyme (HEL), and enzymatic activity is calculated as units of HEL activity (Parry et al., 1965) or HEL equivalents (Karrow et al., 1999).

The primary draw back for measuring lysozyme activity is that a minimum of 20 - 40 µl of sample is required for microtiter assays, a volume not readily available from small fish commonly used in laboratory studies. Moreover, the significance of circulating enzyme activity can be questioned. Plasma or serum lysozyme is bathed in a medium of slightly alkaline pH, which is far above the optimal pH of 5.9 for enzymatic activity. An acidic pH is more typical of the intracellular lysosomal compartment. Based on this difference between the optimal assay pH for circulating lysozyme activity and the actual pH of the serum or plasma sample, one can question whether or not measuring circulating enzyme activity is biologically significant.

Nonetheless, measuring lysozyme activity in fish from small quantities of sample can be problematic. Therefore, an alternative means for quantifying circulating lysozyme is needed. The development of a lysozyme-specific antibody that cross reacts with a variety of
fish species would allow researchers to quantify this important marker of innate immune responses in very small amounts of material.

In addition to characterizing mAb M24-2, this study describes the development and characterization of mAb 2C11, which recognizes a protein found only in highly granulated cells that may be equivalent to eosinophilic granular cells (EGCs) found in various species of fish (Ainsworth, 1992). Since EGCs may function like eosinophils of higher animals (Roszell and Rice, 1998), this antibody reagent may be useful in identifying some of the roles of EGCs in fish-parasite interactions in not only *F. grandis*, but other species of fish as well.

Most studies on fish EGCs have focused on tissues such as intestine, gills and skin (Ellis, 1985; Vallejo and Ellis, 1989; Powell et al., 1991; Holland and Rowley, 1998; Reite, 1998). These tissues are not traditionally considered primary or secondary immune organs of teleost (Zapata et al., 1996), leaving a void in what is known about EGC distribution in lymphoid tissues such as head kidney and spleen. Furthermore, initial studies of EGCs have demonstrated their involvement in parasite responses (Flano et al., 1996; Reite, 1998; Matsuyama and Iida, 1999; Dezfuli et al., 2000; Dezfuli et al., 2004), making these cells a logical focal point in understanding fish immunoparasitology.

Fish innate immune responses are routinely evaluated as indicators of immune function and status following exposure to pathogens, biological response modifiers, immunotoxicants, and nutritional regimes (Zelikoff et al., 2000; Bols et al., 2001; Rice, 2001; Rice and Arkoosh, 2002). The work herein describes the development and characterization of mAbs for detecting, localizing, and quantifying cellular immune responses in fish as a possible indicator of immune status.
Materials and Methods

A monoclonal antibody against fish lysozyme was generated and previously described by Frederick (2004), but until now further characterization and exploration of possible applications of the antibody have not been carried out. Adult mummichogs, *Fundulus heteroclitus*, (8 – 12 grams) were collected with baited minnow traps at the Belle W. Baruch Institute for Marine and Coastal Sciences, Baruch Marine Field Lab, Georgetown, SC USA and transported in aerated coolers to laboratory housing facilities at Clemson University. Fish were transferred to 100 L tanks containing aerated 12 parts per thousand (ppt) artificial sea water (Instant Ocean) and maintained at 27 °C. After seven days of acclimation to laboratory conditions, 50 fish of both genders were sacrificed by lethal anesthesia with 1 g L⁻¹ MS-222 in ambient artificial seawater. Fish were bled from the caudal artery using a single-edged razor blade and capillary action within heparinized hematocrit tubes. The hematocrit tubes were centrifuged, after which the plasma from individuals was collected and stored at -80 °C until further analysis.

*Correlating circulating lysozyme protein with enzymatic activity*

Plasma samples from 50 male and 50 female mummichogs were pooled and used to correlate circulating enzymatic activity with specific protein content, and to determine optimal conditions for both using the same-pooled sample. Preliminary experiments revealed that mAb M24-2 recognized only denatured HEL (boiling the sample for 5 min in dH₂O), however fish plasma did not require denaturing measures (data not shown). Additional experiments revealed that diluting samples and the HEL standard in bicarbonate buffer (pH 9), and not PBS (pH 7.2), nor citrate buffer (pH 5.5), was required for optimal
ELISA conditions (data not shown). Each ELISA plate was coated with 100 µl denatured HEL as serial dilutions in quadruplicate. Forty µl of pooled plasma were added to another set of 4 wells. Twenty µL of plasma from each of these wells were serially diluted in bicarbonate buffer to give equivalent well contents of 20, 10, 5, 2.5, 1.25, and 0.6125 µl plasma per well, each in quadruplicate. Eighty µl of bicarbonate buffer were then added to each well for a total volume of 100 µl, and the plates were incubated at 4 °C overnight. The following morning each plate was washed three times with PBS-TW-20 and blocked for 1 hr with 3% BSA. Each ELISA plate then received 100 µl of mAb M24-2 as confluent hybridoma supernatant and incubated at room temperature (22 °C) for 1 hr, followed by three washings and incubation with goat anti-mouse IgG –AP (Southern Biotechnology, Birmingham AL USA; 1:2000) as the secondary antibody. After a 1 hr incubation period, the plates were washed four times, at which time all wells received 100 µl of a 1 mg ml⁻¹ \( p \)-nitrophenyl phosphate solution in alkaline phosphatase (100mM NaCl, 5mM MgCl₂, 100mM diethanolamine, pH 9.5) buffer. Alkaline phosphatase activity was detected at 405 nm using a microtiter plate reader. Optical density data were then converted to HEL equivalent protein µl⁻¹ using the standard curve provided by HEL-containing wells.

Plasma lysozyme enzymatic activity in the same pooled sample was determined using procedures of Parry et al., (1965) as modified by Burton et al., (2002). Plates were set up exactly as described for the above ELISA, except that 0.05 M sodium phosphate buffer, pH 6.9, was used as the dilution buffer for both HEL and plasma samples. To initiate enzymatic reactions, 180 µl of a 0.2 mg ml⁻¹ stock solution of heat killed Micrococcus lysodietikus (Sigma Chemical Company, Saint Louis MO, USA) in 0.05 sodium phosphate buffer, pH 5.9, were quickly added to all wells of the plate. The reaction was carried out at room temperature.
(22°C) and read at 450 nm at 5 min intervals over a 30 min period, then again at 10 min intervals for another 30 min. One unit of lysozyme activity was defined as a decrease in absorbance of 0.001 min⁻¹.

Development of mAb 2C11 against F. grandis granulated leukocytes

Gulf killifish, Fundulus grandis, were collected from the relatively pristine bayous and marshes of Pass Christian, Mississippi, USA and held in aerated running sea water (15 ppt) during transport to the laboratory holding facilities at the University of Southern Mississippi’s Gulf Coast Research Lab, Ocean Springs, MS. Fish were then packed in oxygen-saturated bags and shipped overnight to Clemson University, where upon they were temporarily held in 200 L tanks containing 15 ppt Instant Ocean and equipped with Fluval 404 recirculating biofilters (Rolf C. Hagen, Inc., Winnipeg, Canada). The water was partially changed on a weekly basis. Killifish were maintained on a diet prepared as described, and fed daily a ration of approximately 5% body weight (van Veld et al., 1987).

Lymphoid tissue and cell preparation

Head kidney (HK) and spleens from thirty killifish were removed and prepared as single cell suspensions (Roszell and Rice, 1998) and layered over discontinous Percoll gradients consisting of 3 mL each of 1.060 and 1.080 g/cm³. Gradients were centrifuged for 25 min at 350 x gravity. Following centrifugation, cells were removed from the interface of each gradient layer, washed (3x) by centrifugation, and resuspended in Lebovitz-15 (L-15) media (Gibco, Gaithersburg, MD USA) and counted using a Neubueer hemocytometer.
Viability was determined by trypan exclusion. The cells were then aliquoted into vials containing 5 x 10^6 cells per vial. The first two vials were mixed with TiterMax Gold Adjuvant (TiterMax, Atlanta, GA USA) following the manufacturer's instruction used in the initial immunizations. The remaining vials were centrifuged to pellet the cells, the media removed by aspiration, and cells frozen as dry pellets at -80°C until subsequent booster immunizations.

Two 6-week-old female RBF/dnj mice (Jackson Labs, Bar Harbor, ME USA) were injected with the freshly isolated lymphoid cells in the above adjuvant. For booster immunizations given on days 14, 35, and 56, the above vials were quickly thawed and the cell pellets were resuspended in 500 µl of PBS, mixed by gentle pipetting and injected into each mouse i.p. (5 x 10^6 cells per mouse) without adjuvant. Mice were sacrificed five days after the last booster immunization, their spleens removed and prepared as single cell suspensions. Cells were then fused with FOX myeloma cells (ATCC, Manassas, VA USA) with polyethylene glycol 4000 (ECM, Newark NJ, USA) at a ratio of 3:1, respectively, in RPMI 1640 media (Gibco) supplemented with penicillin, streptomycin, L-glutamine, sodium pyruvate, HEPES, adenine, aminopterin, and thymidine (each from Sigma, St. Louis, MO USA).

**Hybridoma screening**

Initial screening of hybridomas was performed by ELISA using Immunolon plates (Immulon #2, Dynex Technologies, Chantilly, VA USA) coated with lymphoid cells. Plates were first coated with 100 µl well^-1^ of a 0.01 M solution of poly L-lysine at room temperature for 2 hr. The media was removed and replaced with 100 µl of fresh *F. grandis* lymphoid cells
(5 x 10^5 well^-1) in Leibovitz’s media (L-15 media) (Gibco, Gaithersburg, MD USA) and the plates were incubated for 2 hr at 27^0 C. The plates were then centrifuged for 5 min at 350 x gravity (g) and 100 µl of PBS containing 0.05% glutaryldehyde were then slowly added to each well. After a 15 min period the plates were again centrifuged for 5 min at 350 x g and allowed to sit for another 10 min. The media was removed from each well by aspiration and replaced with a 100 µl of 0.1% Triton-X in HBSS. Plates were allowed to incubate at room temperature for 30 min, at which time the wells were aspirated and replaced with 100 µl of 100 mM glycine-1% bovine serum albumin solution in HBSS and the plates were allowed to sit for another 30 min, after which the solution was removed from the wells. Plates were then washed x 2 with HBSS and x 2 with distilled water, allowed to dry at room temperature, and used for the initial screening of supernatants.

The hybridomas from positive wells were transferred to 24 well plates containing Balb/c thymus feeder cells and allowed to grow to near confluence. Supernatants were then tested for their cross-reactivity with fresh lymphoid cells using flow cytometry to identify granular cells based on side scatter properties and fluorescence intensity after labeling these cells with a FITC-conjugated goat anti-mouse Ig (H+L) antibody. Positive hybridomas were cloned twice by limiting dilution and tested again by flow cytometry, resulting in one hybridoma that secreted an antibody specific for highly granular cells (data not shown).

_Cross-reactivity of mAbs M24-2 and 2C11_

Plasma samples from representative teleostean fishes were used to determine the cross reactivity of mAbs M24-2 and 2C11 among fishes. These samples included striped bass, _Morone saxatilis_, channel catfish, _Ictalurus punctatus_, mummichog, _Fundulus heteroclitus_,
carp, *Cyprinus carpio* and nile tilapia, *Orechromis niloticus*. A solution of HEL in H$_2$O was used as a reference standard. Plasma samples and HEL were diluted in SDS-PAGE 4x sample buffer (40% glycerol, 240mM Tris-Cl pH 6.8, 8% SDS, 0.1% bromophenol blue with 5% (v/v β-mercaptoethanol) and denatured by boiling. Proteins were resolved by SDS-PAGE (4-20%) (BioRad) and transferred to a nitrocellulose membrane. Membranes were blocked (3% BSA with 0.1% gelatin) overnight at 4°C with rocking. Subsequently, the membrane was washed in PBS containing 0.5% Tween-20 (PBS-TW-20) for 5 min. Appropriate primary antibodies, either mAb M24-2 or 2C11, were added for 1 hr, washed (3 X 5 min), and goat anti-mouse IgG with alkaline phosphatase (AP) was added for 1hr. After secondary incubation, blots were washed (3 x 5 min) and AP activity was observed with 5-bromo-4-chloro-3-indoly-phosphate/nitro-blue tetrazolium (NBT/BCIP) (Fisher Scientific) in AP buffer. Immunoblots were scanned and documented with a GS-710 densitometer (BioRad).

*Cross-reactivity of mAbs M24-2 and 2C11 in adherent lymphoid cells of different teleostean fishes*

Anterior kidney tissues were collected from nile tilapia, channel catfish, and mummichog and dissociated in HBSS adjusted to an osmolarity compatible with each species. Channel catfish and tilapia tissues were pushed through an 80 x mesh screen for dissociation, while mummichog tissues were dissociated by repeated pipetting through a 1 ml syringe without a needle. Tissue homogenates were transferred to a 15 ml tube and the debris was allowed to settle by gravity for 2 minutes, at which time the overlying media containing cells was transferred to another tube. Cells were suspended in HBSS, washed x 2 by centrifugation, and counted using a standard Neubauer hemocytometer. Lymphoid cells were allowed to adhere to glass cover slips pre-cleaned with acetone and treated with poly-L-
lysine (1 mg ml⁻¹ in distilled water). Approximately 5 × 10⁵ cells were added to the cover slips in 300 ul of HBSS and allowed to adhere over a 30 min period of incubation in glass Petri dishes. Non-adherent cells were then removed from the cover slips and Petri dish by gentle aspiration with HBSS. The cover slips containing adherent cells were fixed in 3% methanol-free formalin (Polysciences) for 30 min and washed three times at room temperature with PBS-TW-20 while still in the Petri dish. The cells were then permeabilized with 0.1% Triton-x-100 (Sigma) in HBSS over a 10 min period, followed by another three washes. Slides were then blocked with a 3% BSA solution in HBSS for 1 hr at room temperature. After three more wash steps, cover slips were probed with mAb M24-2 or 2C11 as confluent hybridoma supernatants for 1 hr, followed by extensive washing with PBS-TW-20. Slides were then incubated with FITC-conjugated goat anti-mouse IgG (H+L) (Southern Biotechnology, Birmingham AL USA; 1:300) solution for 45 min, followed by extensive washing steps. Controls consisted of slides incubated with an isotype-matched irrelevant mAb and FITC-conjugated goat anti-mouse IgG or secondary antibody alone. Slides were then prepared for confocal or epifluorescence microscopy.

Another portion of the cells from each species of fish were pelleted by centrifugation prior to lysis for detecting specific protein in lymphoid cell lysates. This was also done using F. grandis cells prepared the same way as described above for F. heteroclitus. Cell pellets were lysed in 250 µl lysis buffer (250 mM NaCl, 25 mM Tris-HCl, and 1 mM PMSF, pH 8, with 1% NP-40) for 30 min on ice. The resultant lysates were then centrifuged for 20 min at 14,000 x g. The overlying supernatant was collected and the lysate protein content determined using the Bradford method after a 1:10 dilution in PBS. All samples were then diluted in lysis buffer to a common protein concentration, mixed with SDS-PAGE sample buffer and boiled for 5 min. Cell lysate samples and a HEL standard were then
electrophoresed on a pre-cast 4-20 % SDS-PAGE gel (BioRad, Richmond, CA USA) and transferred to nitrocellulose membranes overnight. Immunoblotting steps using mAbs M24-2 or 2C11 were the same as above for serum samples.

Localization of lysozyme protein and granules in different subpopulations of lymphoid cells

While lysozyme is presumed to originate from macrophages and other phagocytes, studies were carried out to determine if mAb M24-2 recognized a protein in multiple cell types in lymphoid tissues. Anterior kidney and spleens from 3 adult channel catfish (400 – 700 grams) were pooled and homogenized as described above. Cell homogenates were applied to a series of percoll gradients yielding density interfaces of 1.040/1.050, 1.050/1.060, and 1.060/1.080 g/mL. After centrifuging the tubes for 25 min at 350 x g, cells at each interface and the bottom of the tube were collected and washed twice with HBSS. Cells were allowed to adhere to poly-L-lysine-coated glass slides, fixed and permeablized, then probed for antibody reactivity as described above for adherent cells.

Cells recognized by mAb 2C11 are low in number in some species of fish like channel catfish; therefore this approach was not taken as part of the characterization of mAb 2C11.

Immunohistochemistry using mAbs M24-2 and 2C11

The ability of mAbs M24-2 and 2C11 to recognize their specific protein in fixed and embedded tissues was determined using standard immunohistochemical techniques. Head kidneys and spleens from euthanized F. grandis were removed and immediately fixed in 10%
formalin in phosphate-buffered saline (pH 7.4), 70% ethanol or snap frozen in OCT media. Fixed samples were then dehydrated through a graded series of ethanol to 100% ethanol and embedded in Immunobed (Polysciences Inc., Warrington, PA) according to the manufacturer’s directions and cut at 2 µM sections. Sections were hydrated, washed (PBS; 3 x 2 min), and blocked (1% non-immune goat serum in 1% BSA; 15 min). Monoclonal antibody M24-2 was used as confluent hybridoma supernatant and incubated with sections for 1 hr in a humidified chamber. Following incubation, slides were washed (PBS; 3 x 2 min) and incubated with gold-conjugated goat anti-mouse IgG+IgA+IgM antibodies (Ted Pella Inc., Redding, CA) diluted in block solution for 1 h, followed by extensive washing in ddH₂O and then silver enhancement (Ted Pella, Inc., Redding, CA) for 23 min. Sections were stained for 20 sec in 0.5% azure II and 0.25% basic fuschin in a 0.5% Na-borate solution, mounted (Bio-Mount, Ted Pella, Inc., Redding, CA), and photographed. The reactivity and distribution of mAb M24-2 and 2C11 labeled cells was determined using digital photographs taken with a Q Imaging Micropublisher camera mounted on a Nikon Eclipse E600 microscope.

Preliminary studies showed that mAb 2C-11 does not recognize its epitopes in plastic-embedded tissues, therefore frozen sections of lymphoid tissues from the mummichog were used. Spleens snap frozen in OCT media were cut at 8 µm using a Microm HM505E cryostat. Tissue sections were added to Superfrost Plus (Fisher Scientific) slides and immediately fixed in cold acetone for 1 minute. Slide sections were encircled using a Pap Pen (Scientific Device Laboratories) to maintain separate positive and negative staining areas. Sections were rehydrated using PBS (15 min), blocked (10% normal goat serum in PBS), and mAb 2C11 added as confluent hybridoma supernatant to the positive area, while negative area remained in blocking solution for 1 hr in a humidified chamber.
Slides were then washed (3 x 5 min) in blocking solution and a fluorescein-isothiocyanate (FITC) labeled goat anti-mouse IgG (H+L) (Molecular Probes) secondary was diluted 1:100 in PBS and added to slide that were then incubated in the dark for 1 hr. Secondary antibody was removed by washing (3 x 5 min) in PBS. Sections were then counterstained using 4'-6-diamidino-2-phenylindole (DAPI) (2µg/ml in PBS) for 20 min. Slides were then mounted with PBS and viewed using a Nikon Optiphot-2 microscope and SPOT (Diagnostic Instrument, Inc.) camera.

**Results**

Monoclonal antibody M24-2 is an IgG1 κ immunoglobulin that recognizes a protein of ≈14.5 kD in size in mummichog plasma, and it recognizes HEL (Figure 2.1). To examine the degree of cross-reactivity of mAb M24-2 among different species of fish, plasma samples were subjected to SDS-PAGE and immunoblotting to show that this antibody recognizes a similar plasma protein in a broad range of both freshwater and marine fish (Figure 2.3).

One of the most useful applications of mAb M24-2 in fish research is in the development of ELISAs for quantifying lysozyme in a variety of fish, but especially in very small fish from which plasma or serum volumes are too small to perform routine enzymatic assays. However, any ELISA using mAb M24-2 as the detection reagent needs to be as sensitive as enzymatic assays. Therefore, a pooled sample of mummichog plasma was used in both ELISA and enzymatic assays and the data compared (Figure 2.2). Enzymatic data cannot be detected below 5 µl of plasma sample, while ELISA O.D. values are linear between 10 µL and 0.625 µL. Initial data showed that by adding the equivalent of 20 µL of sample to the plates, the ELISA data were no longer linear (data not shown).
Ultimately, the utility of mAb M24-2 in cellular immune responses of fish depends on cross-reactivity among different species of both freshwater and marine fish. Using standard immunoblotting techniques, it became clear that this antibody reagent recognizes lysozyme in cell lysates from the three species examined (Figure 2.4). Localization of lysozyme protein in cells may be important in certain research protocols, therefore efforts were undertaken to determine if mAb M24-2 recognizes lysozyme in fixed and permeabilized adherent cells from mummichog, channel catfish, and tilapia. This antibody recognizes compartmentalized lysozyme in all three species of fish, showing that the protein is localized in intact lysosomes (Figure 2.5).

While the majority of glass-adherent lymphoid cells are considered phagocytes, and are lysozyme positive, it is possible that cells other than phagocytes express lysozyme. Lymphoid cells from both head kidney and spleens of channel catfish were separated over Percoll density gradients following centrifugation to yield layers of cells typically considered lymphocytes, macrophages, granulocytes, and thrombocytes/red blood cells. These cell types were then coated onto poly-L-lysine-treated glass coverslips and probed with mAb M24-2. Those cells known to separate out as phagocytes (macrophages and neutrophils) expressed lysozyme, while lymphocytes did not (Figure 2.6). However, some red blood cells also stained for lysozyme.

Monoclonal antibody is able to recognize its ligand in plastic-embedded tissues (Figure 2.7), and as seen in fractionated cells this antibody recognizes primarily phagocytes, but also some red blood cells.

Monoclonal antibody 2C11 was developed and subsequently isotyped as an IgG3 κ immunoglobulin molecule (Clonotyping Kit; Southern Biotechnology, Inc., Birmingham, AL USA). This antibody recognizes an intracellular protein of ≈ 17 kDa in size, but does not
recognize this protein in plasma, nor does it react with HEL (data not shown). Using permeabilized and glass-adherent lymphoid cells, it was demonstrated that mAb 2C11 recognizes granule contents of highly granulated cells (Figure 2.8), and thus may be a marker for EGCs. The staining pattern of mAb 2C11 in target cells is distinctly different from what can be seen in mAb M24-2-positive cells. However, like mAb M24-2, mAb 2C11 cross-reacts with a similar protein in similar cells of different species of fish (Figure 2.9). Furthermore, the numbers of 2C11-positive cells is much lower than those staining positive for lysozyme, suggesting further that these two antibodies recognize different cell types.

Using immunohistochemical techniques on frozen sections of spleen, it was demonstrated that mAb 2C11 recognizes EGC cells distributed throughout the organ, and that the intracellular staining pattern is the same as seen in glass-adherent lymphoid cells (Figure 2.10).
Figure 2.1. Immunoblot of mummichog plasma proteins. mAb M24-2 recognizes a low molecular weight protein.
Figure 2.2. Comparison of lysozyme quantification methods. ELISA using M24-2 also gives a linear response, but requires less of a plasma volume that the standard lysozyme activity assay.
Figure 2.3. Immunoblot demonstrating cross reactivity of M24-2 in fish serum. mAb M24-2 recognizes hen egg lysozyme (lane 1). M24-2 also recognizes a 14.5 serum protein of mummichog (lane 2), striped bass (lane 3), channel catfish (lane 4), tilapia (lane 5), and carp (lane 6). Lane 7 was blank.
**Figure 2.4.** Immunoblot demonstrating cross reactivity of M24-2 in fish cell lysates. mAb M24-2 recognizes hen egg lysozyme (lane 2). M24-2 also recognizes a 14.5 serum protein of mummichog (lane 3), channel catfish (lane 4), tilapia (lane 5). 15kD molecular weight marker is in lane 1.
Figure 2.5. Fluorescent images of fish head kidney cells showing cross-reactivity of mAb M24-2. (A) Confocal image of channel catfish cells staining positively with M24-2 and a TRITC-labeled secondary. (B) Epifluorescent micrograph of tilapia cells stained positive for M24-2 and a TRITC-labeled secondary. (C) Epifluorescent image of mummichog cells positive for M24-2 labeled with a FITC-secondary. 1000x oil.
Figure 2.6. Schematic showing catfish cells separated over Percoll gradients in a 15ml tube, isolated, probed with mAb M24-2 and stained with a FITC labeled goat anti-mouse secondary. Phagocytes are typically found in the 60/80 interface, and that is where the majority of the M24-2 positive cells were found. Group 1 images taken with epifluorescent microscope. Group 2 are bright field (BF) images of group 1 micrographs.
Figure 2.7. Immunohistochemistry of F. grandis HK using mAb M24-2. (A) Negative control. 100x. (B) M24-2 staining. 100x.
Figure 2.8. Cross reactivity of primary mAb 2C11 in adherent cells from fish head kidneys. All cells stained with a TRITC labeled goat anti-mouse secondary. (A) 2C11 positive channel catfish cell. 1000x oil epifluorescent micrograph. (B) 2C11 positive tilapia cell. 40x epifluorescent micrograph. (C) 2C11 positive mummichog cell. Confocal micrograph.
Figure 2.9. Immunoblot of fish cell lysates probed with 2C11. mAb 2C11 recognized a ~17 kD protein in the cell lysates of *Fundulus grandis* (lane 1), mummichog (lane 2), channel catfish (lane 3), and tilapia (lane 4). 2C11 did not recognize a protein in plasma samples from any fish.
Figure 2.10. mAb 2C11 positive cells in *F. grandis* spleen cryosections. All sections stained with a FITC labeled goat anti-mouse secondary and viewed with a triple pass filter. Negative controls (A) 100x (C) 200x and (E) 400x. MMCs autofluoresce. Positive staining (B)100x (D) 200x and (F) 400x among spleen parenchyma and colocalization within MMCs.
Discussion

Historically, the process of characterizing fish leukocytes has been difficult because the differences between taxa are tremendous, and comparative immunologists have often worked independently using different techniques and terminology. For example, over 16 different types of granulocytes have been described in fish (Ainsworth, 1992; Zapata et al., 1996), though the actual number is much less, and probably on the order of what is seen in higher vertebrates. The actual number of different cells in fish is exaggerated because investigators have used different morphological characteristics and histological methods to identify cell types. In higher vertebrates, a systematic approach for leukocyte identification is based on monoclonal antibody production against cell-specific markers (Goldsby et al., 2003). To date, no cluster of determination (CD) system has been developed for fish, and very few monoclonal antibodies are available that have a high degree of cross-reactivity among fishes. Possessing an antibody that is cross-reactive among fish taxa would be useful in tying together what is known about different species of fish. Fish immunologists face the reality that there is as much difference between rainbow trout and channel catfish, for example, as between mouse and humans. Therefore fish immunologists must rely on monoclonal antibodies generated for a given species, and only a limited number of monoclonal antibodies react with multiple species (Sepulcre et al., 2002). One approach to solving the problem of a lack of cross-reactive antibodies is to generate and select for antibodies against highly conserved proteins.

In this study, two mAbs, M24-2 and 2C11 were developed and shown to be specific for phagocyte proteins. M24-2 recognizes a ≈ 14.5 kD protein in fish plasma, cell lysates, and whole cells, and it consistently recognizes HEL. While this antibody was made using leukocytes from *F. heteroclitus*, it is cross-reactive in all species of fish so far examined.
Furthermore, mAb M24-2 binds specifically to a protein found in cells known to be macrophages and neutrophils, which are the principle lysozyme expressing leukocytes of fish (Zapata et al., 1996). Surprisingly, mAb M24-2 binds to some erythrocytes in lymphoid organ cell preparations and in tissues embedded in plastic. There are no published reports demonstrating lysozyme expression in red blood cells of fish, thus the observation that mAb M24-2 binds to some, but not all red blood cells suggest that either red blood cells produce lysozyme under specific conditions, or that red blood cells may be a carrier and/or sink for plasma lysozyme. Alternatively, lysozyme may act as an opsonin to target effete red blood cells for destruction by macrophages in the spleen. The observation that high levels of lysozyme circulate throughout the vertebrate body despite sub-optimal physiological conditions for enzymatic activity lends support to the speculation that lysozyme serves a physiological function via means other than enzymatic capability (Yousif et al., 1991; Hanif et al., 2004). If so, then it would be more prudent to quantify lysozyme protein in circulation than to measure enzymatic activity, thus an important application of mAb M24-2. As demonstrated in this study, an indirect ELISA using mAb M24-2 is as sensitive as standard enzymatic assays for quantifying lysozyme. Furthermore, measuring lysozyme activity alone may be a misleading endpoint if an enzyme inhibitor is also present in the assay media or sample at hand, when quantifying the specific protein by immunological means would circumvent this problem. Lastly, the finding that mAb 24-2 recognizes lysozyme in plastic-embedded tissues adds a rare member of antibodies to the short list that recognize their target antigens in plastic. Plastic embedded tissues present their cellular and anatomical features better than other preparations such as paraffin or cryopreservation.

Monoclonal antibody 2C11 recognizes a ≈ 17 kDa protein in fish cell lysates, but not plasma, and only in a small population of leukocytes. While granulocyte populations in fish
remain controversial, it is well established that macrophages and neutrophils are the predominant cell type found in fish lymphoid tissues, and other leukocytes such as EGCs comprise a smaller percentage of cells (Ainsworth, 1992; Zapata et al., 1996). Although this mAb was made from *F. grandis* cells, it cross-reacts with all fish species tested to date which further improves its value as a tool for fish immunologists.

One of the most controversial issues in fish immunology is the nomenclature for EGCs. Because of different histological staining techniques used by different investigators, there is the suggestion that EGCs and fish mast cells are one in the same. To date, only tissues such as gill, intestine, and swimbladder have been used to describe fish mast cells, and descriptions of EGCs vs. mast cells in lymphoid tissues has been ignored. From experiments not described in this study, it is clear that mAb 2C11 does not recognize a protein in the human mast cell line HMC-1. Future studies will determine if this antibody recognizes those cells in intestine, gill, or swimbladder historically referred to as mast cells, and if so then EGCs and mast cells may indeed be the same cell. However, if not, then we can infer that EGCs and mast cells are from different lineages and with different function(s), or that fish mast cells are more like EGCs. Lastly, since EGCs may be functionally equivalent to mammalian eosinophils, they may have a role in host immune responses against parasites. In the presence of mast-cell-derived cytokines and the activation of complement, eosinophils degranulate to release several potent factors known to be antiparasitic (Rothenberg and Hogan, 2006). Since mAb 2C-11 recognizes a granule protein, this antibody may be useful in tracking degranulation events in EGCs. Unlike mAb M24-2, mAb 2C11 does not recognize it’s antigen in plastic-embedded tissues, but does so in cryopreserved and fixed tissues.


Gulf killifish, *Fundulus grandis*, were sampled from Simmon’s Bayou in Jackson County, Mississippi, Gulf of Mexico, USA in 2003 and 2004 and examined for endoparasites. Necropsy revealed the nematode *Eustrongylides ignotus*, the coccidian *Calyptospora funduli*, and a digenean metacercaria of unknown species. Head kidneys, spleens and livers from infected fish and noninfected fish were compared to determine any structural and histological differences. Different histologic procedures were employed and compared. All tissues were probed with a lysozyme specific mAb (M24-2) and a granulocyte-specific mAb (2C11) to compare infectivity status with possible changes in phagocyte response. Other than absence or presence of endoparasites, no histological differences or distribution of lysozyme-positive cells or numbers of eosinophilic granular cells were noted. At least in the fish examined herein, it appears as though internal parasites are well-tolerated in this host-parasite relationship.
Introduction

Despite the large body of literature demonstrating that fish harbor a wide variety of both ectoparasites and endoparasites (Ribelin and Migaki, 1975; Zapata et al, 1996) very little is known about fish immune responses following infection. Evidence suggests that both innate and adaptive immune responses are involved in host-parasite relationships in all vertebrates (Jones, 2001). Nevertheless, many aspects of immunity to parasites in fish have yet to be described.

There are several well-established models for examining the immune responses of fishes to parasites. Ectoparasite models include the monogeneans *Gyrodactylus* spp. and *Dactylogyrus* spp. and the copepod *Lepeoptheirus salmonis*, which have been studied almost exclusively in salmonids (Jorgensen et al., 2007). Haematozoic parasite models consist of the Kinetoplastids’ *Cryptobia* spp. and *Trypanosoma* spp and their infectivity of trout, salmon and carp (Ardelli and Woo, 1998). However, detailed knowledge of histiozoic and coelozoic parasites of fish is lacking. The best-known models of histiozoic and coelozoic parasites of fish are *Myxobolus cerebralis*, the source of whirling disease in salmonids, and *Ceratomyxa shasta*, another myxosporean infecting salmonids (Jones, 2001). The effects of another histiozoic parasite, the digenean trematode *Diplostomum spathaceum*, have been studied immunologically in rainbow trout. There are no well-characterized nematode models for fish immunoparasitology, and most of the fish models are salmonids. It is therefore important to further the current understanding of immune responses of fish to parasites by expanding not only the types of parasite studied, but the type of fish mounting the response as well.

Gulf killifish are small estuarine fish that inhabit the shallow coastal marshes of the Gulf of Mexico from Florida to Texas, and is one of the most abundant fish species found
in high marsh habitats (Solangi and Overstreet, 1980). Several studies demonstrate the utility of *F. grandis* as a model for fish immunology and immunotoxicology (Nevid and Meier, 1993; Nevid and Meier, 1994; Nevid and Meier, 1995; Roszell and Rice, 1998; Rice and Xiang, 2000) and the observation that *F. grandis* are heavily colonized by endoparasites (Chapter 1) support the use of *F. grandis* as a good model for furthering out understanding of host-parasite relationships in fish.

*Eustrongylides ignotus* is a histiozoic nematode whose definitive hosts include piscivorous Ciconiiformes such as herons and egrets. *E. ignotus* infection causes large scale die-offs in nesting Ciconiiformes. Adult birds shed nematode eggs into the water in feces and the eggs are in turn consumed by an aquatic oligochaete, the first intermediate host. Numerous fish species can acquire *E. ignotus* infection by ingesting an infected oligochaete, thus becoming a second intermediate host to the nematode (Coyner et al., 2003).

*Calyptospora funduli* is an apicomplexan parasite that infects numerous species of Cyprinodontidae fishes included *Fundulus grandis, F. heteroclitus, F. similis, F. puverens, F. jenkinsi, F. confluentus* and the Atherinidae *Menidia beryllina* (Fournie and Overstreet, 1993). Although *Calyptospora funduli* infection of *F. grandis* has been well characterized (Fournie et al., 2000), host-parasite interactions of apicomplexan infection of fish in general have been poorly described (Jones, 2001).

In the study described herein, a population of *F. grandis* from Simmon’s Bayou, Jackson County MS was examined over a two year period. Fish were necropsied and examined for presence of parasites, and their head kidney, spleen and livers were prepared for histological examination. Larvae of *E. ignotus* were found in the coelom, and after histologic sectioning *C. funduli* and *E. ignotus* were detected in liver tissue, and an unidentifiable digenetic trematode metacercaria was observed in the head kidney and liver.
Tissues were examined for their architecture as well as the abundance and degree of infiltration of phagocytes using monoclonal antibodies 2C11 and M24-2, which recognize fish granulocyte protein and fish lysozyme, respectively (Chapter 2).

**Materials and Methods**

*Collection and Fish Necropsy*

Adult Gulf Killifish, *Fundulus grandis*, were collected from Simmon’s Bayou, Jackson County, MS USA and shipped overnight to laboratory housing facilities at Clemson University. Fish were transferred to 100 L tanks containing aerated 18 parts per thousand artificial sea water (Instant Ocean) and maintained at 25 °C. After three days of acclimation to laboratory conditions, *F. grandis* were anesthetized with 1 g/L tricaine methane sulfonate (MS-222) in ambient artificial seawater, weighed, measured, sexed and observed for any obvious external signs of infection. Fish were then bled and decapitated, then eviscerated and examined for the presence of endoparasites (Chapter 1).

In addition to any endoparasites present, fish head kidneys, spleen and liver were collected during this study and fixed in neutral buffered formalin (NBF), 70% ethanol (EtOH), or snap frozen in O.C.T. media. Only worms and livers were collected from the first sampling period. Head kidneys and spleens were used for studies in Chapter 2. Samples fixed in NBF or 70% EtOH were dehydrated using graded progressions of ethanol, cleared with Xylenes, and returned to 100% EtOH to await embedding. Tissues were then preinfiltrated with a 50:50 solution of Immunobed (Polysciences, Warminster, PA)
overnight, infiltrated in 100% Immunobed overnight, embedded in size appropriate molds, and placed in a dessicator for at least 4 days before microtomy.

*Immunohistology*

Samples embedded in Immunobed were removed from a dessicator for microtomy, and multiple 2µm sections were cut of each sample using a Leica RM 2165 microtome, floated in a water bath and collected on labeled slides. Four tissue sections were adhered to each slide with two on one end and two on the other as an appropriate way to orient tissues for immunohistochemistry (IHC). Some samples were stained with 0.5% azure II and 0.25% basic fuchsin, dipped in Xlenes and cover slipped with Cytoseal XYL (Richard-Allan Scientific, Kalamazoo, MI) to observe normal tissue structure.

Two areas of two tissue sections each were oriented on the slides and encircled with a Pap Pen (Scientific Device Laboratories, #9804) to separate positive and negative controls. Slides were then rehydrated by dipping into 100% EtOH for two minutes and then into deionized water (DI H$_2$O) (2 x 2 min). Slides were then placed in a humidified chamber and blocked with blocking buffer (1% non-immune goat serum, 1% bovine serum albumin in phosphate buffered saline, pH 7.2) for 15 min. Specific primary antibodies were added as confluent hybridoma supernatant on the encircled region of the slide designated as positive. On the other area of the slide, designated as negative, blocking solution was added for an hour in the humidified chamber. After an hour incubation, slides were flicked again and washed with PBS (3 x 5 min). Gold-conjugated goat anti-mouse IgA + IgG + IgM secondary antibody (Ted Pella, #15985) was diluted 1:100 in blocking solution and added to each slide in chamber. After another hour incubation, slides were washed in running DI
H₂O for at least five minutes and then in a separate container with DI H₂O for at least another five minutes with constant dipping agitation. Slides were then silver enhanced for 23 minutes and again, washed in running DI H₂O for at least five minutes. Finally, slides were stained and cover slipped as stated above and ready for imaging. Micrographs were taken using a Q imaging Micropublisher camera mounted onto a Nikon Eclipse E600 microscope.

Tissues snap frozen in OCT media were cut at 8µm using a Microm HM505E cryostat, adhered to Superfrost Plus (Fisher Scientific) slides with warmth from a finger underneath the slide, and dropped in acetone for fixation for one minute. Slides were then air-dried and sections were encircled using Pap Pen in same orientation as mentioned above with a positive and negative area. Sections were rehydrated by adding PBS (3 x 5 min) to enclosed tissue areas and gently tapping slides on a paper towel for fluid to be removed by capillary action. This type of fluid removal was necessary and used during all steps as cryosections can become uplifted from slides fairly easily and wash away. Next, a blocking solution (10% nonimmune goat serum in PBS) was added for 15 minutes in a humidified chamber. After blocking, select primary antibodies were added as confluent hybridoma supernatant on the encircled region of the slide designated as positive, blocking solution was added to the negative region, and slides were incubated for an hour in the chamber. Primary antibody was removed by washing with blocking solution (3 x 5 min). Fluorescein isothiocyanate (FITC) labeled goat anti-mouse IgG (H+L) (Invitrogen- Molecular Probes; #F2671) was diluted 1:100 in PBS, added to each slide, and incubated in the dark for an hour. FITC labeled secondary was removed by washing three times for five minutes each with PBS and counterstained with 4’-6-Diamidino-2-phenylindole (DAPI) at a concentration of 2 µg/ml in PBS for 20 minutes. Slides were then rinsed gently with PBS, cover slips mounted with PBS, and were ready for immediate imaging using a triple pass filter.
Micrographs were taken using a Nikon Optiphot-2 microscope and SPOT (Diagnostic Instruments, Inc.) camera.

Melano-Macrophage Center Counts

Stained spleens and head kidneys were examined for the number of melano-macrophage centers (MMCs) following the procedures of Camp et al. (2000) with a little modification (Camp, Wolters et al. 2000). Head kidneys and spleen from each fish collected were observed at 100x magnification and MMCs were counted in ten different fields and averaged.

2C11-positive cell counts in spleen cryosections

Spleen cryosections were stained with 2C11 as described above for immunofluorescence. Six micrographs of spleen tissues at 10x were captured. Using the computer program associated with the SPOT camera to visualize captured images, a 100mm x 15mm Petri dish was placed against the spleen micrographs on the computer in ten different areas, and the number of positive cells visualized within the Petri counted and averaged.

Fatty Liver Scores

Each stained liver was photographed at 40x and all of the lipid droplets in a defined area were counted. The count was preformed on ten different defined areas from each liver
and averaged. Livers were given a score from 1-5 based on the amount of lipid counted in the designated area with 1 being the lowest amount of lipid and 5 being the highest.

Correlating Infection Statistics

Correlation analysis between fish sex and size and specific parasite load were carried out using GrafPad Instat-3 software. Information was also gathered to determine if specific parasite load was correlated with the load of any other parasite and if parasite load was related to the number of MMCs per organ, the number of 2C11-positive cells in spleen and the fatty liver score.

Results

Normal F. grandis tissue structure

Fundulus grandis head kidney structure consists of both renal and hematopoietic tissues (Figure 3.1). Renal components of the head kidney include the nephron and collecting ducts. The nephron is composed of a glomerulus surrounded by Bowman's capsule, followed by a series of tubules (Figure 3.2). The glomerulus is a tuft of capillaries, and as mentioned, is enclosed by Bowman's capsule, a layer of simple squamous epithelium (Figure 3.2.A). Proximal convoluted tubules are composed of simple columnar epithelium with a brush border (Figure 3.2.B) and are extremely prominent throughout the head kidney. Distal convoluted tubules (DCT) are made of simple columnar epithelium but lack microvilli (Figure 3.2.C) and are less prominent. The cytoplasm of DCTs was found to stain intensely
acidophilic and have basally located nuclei. Collecting ducts are large tubules with tall columnar epithelium (Figure 3.2.D).

Hemopoietic tissue of the head kidney is typified by a dense cellular network of many undifferentiated stem or blast cell, erythrocytes, leukocytes and vasculature (Figure 3.3.A). Melano- macrophage centers (MMCs) are scattered throughout the hematopoetic stroma (Figure 3.3.B and 3.3.C). MMCs contain discrete groups of pigmented macrophages.

Spleen tissue in *F. grandis* is composed almost entirely of red pulp with diffuse areas of leukocytes and MMCs (white pulp) scattered throughout the stroma (Figure 3.4.A). These areas of leukocytes are usually associated with vasculature (Figure 3.4.B and 3.4.C). The spleen is surrounded by a thin connective tissue capsule.

*F. grandis* liver is not organized in hexagonal lobes as is seen in mammalian liver (Figure 3.5.A). The organization is a complex network of vessels (3.5.C), sinusoids and hepatocyte cell cords. Hepatocytes varied from fish to fish with respect to polyhedral shape, cytoplasmic staining and lipid vacuolization. Hepatic triads were present (Figure 3.5.B). Bile duct could be differentiated from hepatic artery and vein by presence of simple cuboidal to columnar epithelium with basally located nuclei. Intra hepatopancreas tissue (Figure 3.5.D) is spread throughout liver.

Hepatopancreatic tissue could be found located within or outside of liver parenchyma proper. Intra hepatopancreas tissue was usually associated with a vessel (3.6.A). Extra hepatopancreas tissue was located close to the liver and also associated with vasculature (3.6.B). Pancreatic acinar cells are extremely basophilic and contain eosinophilic granules. Endocrine hepatopancreatic islet cells are lighter staining less pronounced than their exocrine counterparts (Figure 3.6.C).
Abnormal histologic features

In observing these tissues for normal histologic features, we detected parasites in some head kidney and livers. No parasites were observed in the spleen. All infected tissues were sent to Dr. Jack Fournie, EPA-Gulf Coast Division, for identification of parasites. All livers from this study were from the first collection date and Table 3.1 summarizes the prevalence, intensity and location of all organisms found in these livers. The apicomplexan *Calyptospora funduli* was observed in all livers sampled (Figure 3.7.A-D) and was grouped into large pockets of spores or spread diffusely throughout liver parenchyma. An immature (L2 or L3) *E. ignotus* was detected embedded in one liver (Figure 3.8A-B). An unidentifiable digenetic trematode metacercaria was also found in one liver (Figure 3.7.A) and in numerous head kidneys. All head kidneys observed were from the second collection and Table 3.2 reviews the prevalence, intensity and types of parasites found from this collection period. Metacercaria were typically distributed around periphery of head kidney and surrounded by a connective tissue capsule (Figure 3.9A-D). In higher vertebrates, eosinophils are known as the major effector cell against parasitic worms, but there was no obvious collection of eosinophil-like cells, or any other particular cell type, around the periphery of the cysts.

Immunohistochemistry

Immunohistochemical staining of tissues using mAb M24-2 was successful in plastic. Figure 3.10 illustrates both negative and positive staining of head kidney tissues. Positive staining is indicated by small silver particles in the cytoplasm. Cells staining positive for M24-
2 appeared to include some erythrocytes and some larger leukocytes (Figure 3.10.D). None of the renal tubules or connective tissues stained positively with M24-2.

Spleen and liver tissues were also probed with mAb M24-2 (Figure 3.11 and 3.12). As seen in the head kidney, some erythrocytes and leukocytes stained positively, but hepatocytes, connective tissue, muscle and hepatopancreas did not.

Head kidneys infected with trematode metacercaria were probed with M24-2 to determine if there was an accumulation of M24-2 positive cells (Figure 3.13) around the cyst, but there was no such accumulation observed. Staining pattern was determined to be random.

IHC was also performed on head kidneys and spleens using mAb 2C11 in plastic (Figure 3.14). Unfortunately, 2C11 did not seem to bind its epitope in plastic in either tissue.

Melano-Macrophage Center Counts

MMCs were counted from head kidneys and spleens of all fish and compared to the loads of all parasites per fish and per organ to determine if there was a correlation between MMC number and parasite load (Table 3.3). There was no correlation between MMC number and parasite burden.

2C11-Positive Cell Counts

Although 2C11 does not appear to work in plastic, the antibody stained cells in spleen cryosections (Figure 3.15). 2Cll positive cell counts were compared to load of all
parasites found to determine if this cell type was upregulated during parasitic infection. There was no correlation between parasite load and 2C11 cell number.

_Fatty Liver Score_

A score was assigned to the liver of each fish collected based on the amount of lipid droplets counted and averaged in a defined area (Table 3.4). The assigned score for each liver was compared with parasite load to determine if there was a correlation between the fattiness of the liver and parasite burden and no correlation was found.

_Correlation statistics results_

Statistics were preformed to determine if there was any correlation between and among fish size and sex and factors such as specific parasite load, number of MMCs in head kidney and spleen, 2C11 positive cells. We found no correlation among any of the parameters examined.
Figure 3.1. Typical *F. grandis* head kidney tissue at 40x (A) and 100x (B). Note the renal tubules (arrows) and hematopoetic regions (arrowheads).
Figure 3.2. Renal structures of *F. grandis* head kidney tissue. (A) Glomeruli (arrows) enclosed by Bowman’s capsule (arrowheads). 200x. (B) Distal convoluted tubules lack brush border. 600x. (C) Proximal convoluted tubules with brush border. 400x. (D) Large collecting duct. 100x.
Figure 3.3. Hematopoetic regions of *F. grandis* head kidney tissue. (A) Large area of hematopoetic tissue with melano- macrophage centers (MMCs) (arrows) 100x. (B) MMC from *F. grandis* tissue fixed with 70% ETOH. 400x. (C) MMC from *F. grandis* tissue fixed with NBF. 400x.
Figure 3.4. *F. grandis* spleen. (A) Spleen at 20x with MMCs scattered throughout. Organ almost entirely red pulp with thin connective tissue capsule. (B) Capillary (arrow) with erythrocytes beside a MMC from tissue fixed in 70% ETOH. 400x. (C) Spleen with red pulp and MMCs from tissue fixed in NBF. 400x.
Figure 3.5. *F. grandis* liver tissue and associated structures. (A) Low mag view of liver with large vessels and ducts throughout. 20x. (B) Hepatic triad composed of hepatic artery, hepatic vein, and bile duct (arrow). 100x. (C) Large hepatic vein. 20x. (D) Small foci of hepatopancreas (arrow). 100x.
Figure 3.6. *F. grandis* hepatopancreas. (A) Low power view of intrahepatopancreas embedded within parenchyma. 100x. (B) Extra hepatopancreas. 100x. (C) Inset of B; hepatopancreatic tissue surrounds lighter staining artery. Basophilic exocrine pancreas acinar cells with eosinophilic granules (arrow). Endocrine islets stain lighter (arrowhead). 400x.
### Table 3.1. Prevalence and intensity of endoparasites of *F. grandis* from 12/11/03 collection. P = prevalence; I = intensity

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<th>Digenetic Trematode in liver</th>
<th>C. funduli in liver</th>
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Figure 3.7. Apicomplexan *Calyptospora funduli* infection of liver. (A) Low power image of liver with *C. funduli* (box) and digenetic trematode metacercaria (arrow) infection. 20x. (B). Inset of A; higher power view of *C. funduli* spore pocket. 400x. (C) Different organization of *C. funduli* in liver. Spores either arranged in pockets (circle) or scattered (arrows) diffusely throughout parenchyma 100x. (D) Pocket of parasite separate from tissue, but blend easily with fattier livers. 100x.
Figure 3.8. *E. ignotus* larvae encysted in liver. (A) Low power micrograph of liver section with *E. ignotus* larvae in upper right quadrant. 20x. (B) Higher power view of cyst sectioned through gonad region. 40x.
### Prevalence and Intensity of Parasites in *F. grandis*- 07/01/04

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**Table 3.2.** Prevalence and intensity of endoparasites of *F. grandis* from 07/01/04 collection. P = prevalence; I = intensity
Figure 3.9. Digenetic trematode metacercaria in *F. grandis* head kidneys. (A) and (B) Low power micrographs of metacercaria in two different head kidneys. Metacercaria typically found at periphery of tissue. 20x. (C) Higher magnification of metacercaria in head kidney tissue. 100x. (D) Inset of (C); Metacercaria encysted by fish fibroblasts; no obvious accumulation of white blood cells at margin of cyst.
Figure 3.10. Immunohistochemistry of *F. grandis* head kidney using mAb M24-2 in plastic and a gold-conjugated secondary antibody. Tissue was silver enhanced. (A) Low power image of a negative control (did not receive primary antibody) head kidney. 100x. (B) Same head kidney treated with mAb M24-2. 100x. (C) Higher power image of a renal tubule from (A). 600x. (D) Higher power image the same renal tubule as (C) treated with M24-2. 600x.
Figure 3.11. Immunohistochemistry of *F. grandis* spleen using mAb M24-2 in plastic and silver stained. (A) Negative control spleen. 40x. (B) Spleen stained with mAb M24-2. 40x. (C) Negative control spleen. 400x. (D) Spleen stained with mAb M2401. 400x.
Figure 3.12. Immunohistochemistry of *F. grandis* liver using mAb M24-2 in plastic and sliver stained. (A) Phagocyte (arrow) in negative control. 600x. (B) Phagocyte (arrow) M24-2 positive. 600x.
Figure 3.13. Immunohistochemistry of *F. grandis* head kidney using mAb M24-2 in plastic. (A) Low power micrograph of negative control. Note metacercaria (arrow) in bottom region. 40x. (B) Low power micrograph of M24-2 staining. 40x. (C) Higher power view of head kidney tissue surrounding metacercarial cyst (negative control) and (D) M24-2 staining at 200x. There are no obvious deposits of M24-2 positive cells surrounding cyst.
Figure 3.14. Immunohistochemistry of *F. grandis* head kidney and spleen using mAb 2C11 in plastic. A gold-conjugated goat anti-mouse was used as a secondary antibody and sections were silver enhanced. (A) Negative control spleen. 400x. (B) 2C11 stained spleen. 400x. (C) Negative control HK. 400x. (D) 2C11 stained HK. 400x.
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Table 3.3. Numbers of MMCs in head kidney and spleen along with parasite burden for each fish. These numbers were used for correlation statistics.
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**Table 3.4.** Fatty liver score assignments and parasite burden. A score of 1 being least lipid and 5 the most lipid.
Figure 3.15. Immunofluorescence of spleen cryosections probed with mAb 2C11 and viewed with triple pass filter. (A) Positive spleen. 200x. (B) Negative control. 200x. (C) Positive spleen 400x. (D) Negative control. 400x. Note autofluorescence of MMCs.
Discussion

Normal F. grandis tissue structure

Like most teleosts, *F. grandis* head kidney is composed of nephrons and hematopoietic tissue (Yasutake and Wales, 1983; Zapata et al., 1996; Reimschuessel, 2001). However, distinctions in teleosts nephron structure exist, particularly when comparing freshwater and marine species. These differences are due to the demands of different osmotic environments. There are three major classifications of teleosts nephrons: marine glomerular, marine agglomerular, and freshwater glomerular; none of which are exact models for an estuarine species such as *F. grandis* (Reimschuessel, 2001; Yasutake, 1983). The hematopoietic tissue of *F. grandis* is similar to the estuarine cardinal fish (Fischelson, 2006), and salmonids (Yasutake, 1983), as it is composed of stromal cells, granulocytes, stem cells, erythrocytes, MMCs, and lymphocytes.

Functionally, the teleost spleen serves as secondary site for hematopoiesis and a place for erythrocyte storage and destruction (Yastuake, 1983; Zapata et al., 1996). This is mirrored structurally in *F. grandis* and other teleosts as the spleen is composed mostly of red pulp with diffuse white pulp. The teleost spleen differs from mammalian spleens in that the red and white pulp are not very distinct from one another and teleost have a much thinner connective tissue capsule (Yastuake, 1983; Fishelson, 2006).

The structure and function of the liver of *F. grandis* and most other teleosts does not differ greatly from that of higher vertebrates. Hepatocytes arranged in cords and embedded in a matrix of sinusoids and vessels were observed in this model, and this cellular organization is typical of teleost livers. As seen in most teleosts, *F. grandis* have intra- and
extra-hepatopancreatic tissues that have both endocrine and exocrine function. The exocrine component is composed of basophilic acinar cells containing eosinophilic zymogen particles. The endocrine portion is composed of lighter staining islet cells whose function is to produce insulin and glucagon (Yasutake, 1983; Chapman, 1981; Fischelson, 2006).

Abnormal staining of tissue

There was no observable accumulation of M24-2 staining cells around periphery of metacercaria in the head kidney if infected fish, nor were there deposits of M24-2 positive cells around any parasite in livers. It appears that macrophages are not activated by the presence of metacercaria, *E. ignotus* larvae, and *C. funduli*. Monoclonal antibody 2C11 recognized cells in cryosections of spleen, but not noticeably in plastic sections. This may be due to epitope masking by the fixation procedures or that the antibody cannot reach its epitope through the plastic. While staining of 2C11 head kidney cryosections gave mixed results (data not shown), 2C11 staining patterns were similar in all fish examined. This is probably due to the fact that the teleost spleen contains numerous granulocytes, particularly neutrophils and eosinophils (Fishelson, 2006).

Melano-macrophage centers have been used in numerous studies to determine the health of the fish due to both environmental contaminants and pathogens (Iwama et al. 1996, Camp et al, 2000; Fournie et al, 2001; Agius and Roberst, 2003; Fishelson, 2006). Camp et al. (2000) found that channel catfish, *Ictalurus punctatus*, resistant the bacterial pathogen *Edwardsiella ictaluri* had significantly more MMCs in the spleen than did susceptible fish. However, in regards to environmental contaminants, Fishelson (2006) found that
cardinal fish from a polluted site had more MMCs in spleen than from a reference site. In this case, MMC numbers were not associated with parasite infection.

**Histologic technique**

One of the aims of this study was to compare the different fixative and embedding media effects on immune staining and organ presentation. As mentioned, organs fixed in NBF and 70% EtOH were subsequently embedded in Immunobed. Tissues fixed in plastic can be cut at very thin sections, down to 0.25µm, and are far superior to those embedded in OCT media which can only be cut at 5µm by skilled histologists. Even then, much of the morphology of the tissue is not well preserved. In addition, the samples fixed in NBF had better preserved structure than those in 70% EtOH. Moreover, NBF provided better color contrast of staining. For example, in comparing the staining of NBF fixed tissues (Figure 3.3.C and 3.4.C) to those fixed in 70% EtOH (Figure 3.3.B and 3.3.C.), the tissues fixed with NBF have much greater cytoplasm and nuclear stain affinity than those tissues preserved in 70% EtOH.

However, the drawback of fixing tissues with any type of fixative and then embedding them in plastic is that one risks the cross linking of proteins and the subsequent masking of epitopes which is problematic if conducting IHC. In this experiment, mAb M24-2 works well in plastic, but mAb 2C11 does not.

There is a need for characterizing normal fish tissues in fish health studies, whether it is ecotoxicological or pathogen-related. With the development of cost effective methacrylate embedding media such as Immunobed, tissues historically characterized in paraffin could be redescribed in much more detail. This would be useful for today’s fish biologists as the
quality images of organs altered by pathogen or environmental contaminants are rather poor in the literature.
Literature Cited


CLOSING REMARKS

This dissertation provides baseline information on a host-nematode interaction in fish. Some of the most important results of this study include the first histological examination of normal Fundulus grandis head kidney, spleen and liver and these tissues infected with endoparasites. This work is the first to describe the histologic features of Eustrongylides ignotus. The micrographs presented herein can serve as standards for future studies, including those outside the realm of immunology. In plastic-embedded tissues, M24-2 did not appear to bind to the cuticle of the worm, which was not unexpected as lysozyme is known to be bactericidal, not anti-helminthic. In addition, mAb 2C11, an antibody that recognizes a granulocyte protein in cells that may have some function in protection of higher vertebrates against parasites, did not work in plastic and results from immune staining of worms in cryosections were extremely variable. However, I tested only two antibodies in E. ignotus sections; therefore, different antibodies should be used in the future to determine other host responses.

Monoclonal antibodies M24-2 and 2C11 hold promise for future research in fish immunology. The fact that these antibodies recognize specific proteins in all fish tested to date implies they could be used as important markers in multiple immunological studies across diverse fish taxa, thus possibly bridging isolated fish models and providing clarity to the “confusing” state of fish leukocyte nomenclature. Currently, attempts to immunoprecipitate proteins specific to M24-2 and 2C11 are ongoing, and, providing these antibodies are amenable to this technique, should offer definitive information regarding their respective ligands.
Monoclonal antibody M24-recognizes what I believe to be lysozyme. However, mAb M24-2 recognizes some, but not all fish erythrocytes. It may be that fish erythrocytes serve to transport lysozyme in circulation. It is also possible that M24-2 recognizes a conserved protein found in phagocytes and erythrocytes that is not lysozyme. On the other hand, it is possible that fish erythrocytes are capable of producing lysozyme. If such is true, then this is a significant discovery for non-mammalian immunobiology (as most mammalian erythrocytes are anucleate), and one that will lead to many exciting experiments.

Monoclonal antibody 2C11 may be used in the future to provide clarity to the fish granulocyte debate, at least on the eosinophil front, and hopefully improve the current nomenclature for fish leukocytes. It will be interesting to test 2C11 against tissues of fish previously examined for EGCs/mast cells such as gills, intestine, swim bladder and skin to determine if 2C11 positive cell numbers correlate with EGC/mast cell numbers.

While no significant morphological or immunological endpoints were statistically significant between infected and non-infected fish, there are other aspects of immunity that could be examined in the future, including the presence of immunoglobulins, acute phase proteins or complement if specific antibodies for these proteins become available. Though I did not find differences in the immune parameters examined, others differences may indeed exist.

If the fish are simply tolerant of the worms, the following are some mechanisms by which this tolerance could be achieved. Tolerance to parasites in fish could arise by several mechanisms, as discussed by Glenn Hoffman, in The Pathology of Fishes. Helminths can cause hyperemia, hemorrhage, cellular infiltration of infected tissues, hyperplasia, fibrosis, calcification and necrosis. In other situations however, fish may invest initial energy in
delayed-type hypersensitivity responses that would result in formation of a fibrous capsule/cyst around the worm. Helminth larvae that migrate rapidly to a final site of development (e.g., from the gut to the peritoneum, like *E. ignotus*) and settle to tissues for complete development tend to become encysted. There are no published studies describing how *E. ignotus* develops in a fish host other than what larval stage(s) are usually present within the fish. However, compared to *E. ignotus*’s development in the definitive piscivorous bird host, which involves extensive migration of adult worms resulting in gross inflammation and destruction bird organs, *E. ignotus* seems to be stationary in the fish host because raised tunnels and tissue destruction are not evident. Therefore, the encapsulation scenario seems plausible. Encapsulation would prevent the worm from moving around and penetrating and causing damage and inflammation in other tissues. Also, the cyst would be made of "self" tissue so the fish's immune system would ignore it and be free to engage other pathogens. Other than the mechanical pressure exerted on adjacent host tissues, the fish would seem relatively healthy. This type of encystment in *F. grandis* infected with *E. ignotus* is routinely observed. However, as shown in this study, the number of encysted worms per fish is variable, and rarely are all worms encysted. This may be due to the fact that the nematode only recently penetrated the gastrointestinal tract. This initial energy investment into encysting the parasite seems to be payoff in the case of *E. ignotus* and *F. grandis*, as many of the females examined were gravid, so the energy required to handle the worms was not so great as to render the fish incapable of reproducing.

Additionally, simply put, nematodes are just so big! Because of their size, particularly in this model where one *E. ignotus* larvae was longer than the host *F. grandis*, and one encapsulated worm can be over half the size of the liver, the fish host may be so overwhelmed by the parasite that they immunologically ignore the worms. No mechanisms
It has been established as to how animals ignore large worms, but it has been well documented that nematode size plays a role in tolerance. Many worms live in humans for years without any clinical presentation. In essence, the worms are just too large for a cellular defense to destroy or phagocytize the parasite without extensive damage to host tissues. Strong immune responses are energetically expensive and are associated with physiological perturbations; therefore, as long as the worm is not actively secreting toxins or migrating to cause tissue damage, the host tolerates the parasite.

Immunosuppression is an almost universal feature of parasitic infection(s). Active suppression of a fish immune response by a nematode is not the same as tolerance, but it may offer another mechanism to explain why the worms are not damaged, and why there are no outward signs of inflammation. There is an overwhelming amount of literature on the subject of parasite-directed immunosuppression of their hosts, but mostly from mammalian models. For example, *Heligmosomoides polygyrus*, a nematode of the intestinal tract in mice, secretes an immunosuppressant that renders macrophages incompetent as APCs, although the mechanism is not well understood. Also, it is possible that the worm is secreting something to evade detection by the fish host. Some examples from other animals include *Nippostrongylis brasiliensis*, a worm that secretes an acetylhydrolase that blocks neutrophil chemotaxis by hydrolyzing platelet activating factor (PAF), a very potent chemotaxin.

*Dirofilaria immitis*, the nematode that causes heartworms, have Ig-cleaving proteases on their surface that cleave immunoglobulins before they are able to bind. If the immune system does not find the antigen it was initially recruited to encounter, it should not elaborate effectors indefinitely, and thus tolerance of the worm may occur.

It makes more sense that the fish just spend the initial energy to encapsulate the worm and tolerate the parasite, rather than the worm actively suppressing the immune
system. After all, the ultimate “goal” of *E. ignotus* in these killifish is to reach a definitive bird host so that sexual reproduction of the worms can occur. The capsule surrounding the worms can easily be digested away the digestive tract of the bird; thus the worm can conserve energy for its own growth and reproduction in the definitive host, as opposed to actively suppressing the fish response.

In addition to the above mentioned considerations, it would also be interesting to collect more *F. grandis* and fix all available tissues to determine more typical histology of these tissues, to conduct more immune staining, and to examine individuals for total parasites to get an even clearer picture of the load placed on these killifish.

One means to determine whether the worms are being tolerated by the fish, or whether or not they are actively suppressing the immune function of their hosts, is to conduct a pathogen challenge. A tank study set up in which fish from Simmon’s Bayou were randomly separated into tanks and challenged with either an environmentally relevant pathogen, such as *Vibrio* spp., or even an immunotoxic xenobiotic, would be telling. From an ecological point of view, it might be prudent to determine if the same parasite fauna exists within the fish post hurricane Katrina.