Winter 2018

Lack of Delayed-Type Hypersensitivity in Striped Bass (*Morone saxatilis*)

Jessica Shannon Miller
Old Dominion University, jmill159@odu.edu

LACK OF DELAYED-TYPE HYPERSENSITIVITY IN STRIPED BASS

(*MORONE SAXATILIS*)

by

Jessica Shannon Miller
B.S. May 2014, Old Dominion University

A Thesis Submitted to the Faculty of
Old Dominion University in Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

BIOLOGY

OLD DOMINION UNIVERSITY
December 2018

Approved by:

David T. Gauthier (Director)

Dan Barshis (Member)

Wolfgang Vogelbein (Member)

ABSTRACT

LACK OF DELAYED-TYPE HYPERSENSITIVITY IN STRIPED BASS (*MORONE SAXATILIS*)

Jessica Shannon Miller
Old Dominion University, 2018
Director: Dr. David T. Gauthier

Mycobacteriosis is a fatal disease in fishes caused by acid-fast bacteria in the genus *Mycobacterium*. This disease impacts aquaculture, aquariums, and wild fishes. Unfortunately, there are currently no non-lethal diagnostic tests for mycobacterial infection in fishes. Type IV delayed type hypersensitivity (DTH) responses *in vivo* and *in vitro* are currently used for the non-lethal detection of mycobacterial infections in humans and in animals; however, there is little information available on DTH responses in fishes. In this work, we examine *in vivo* DTH response in Striped Bass (*Morone saxatilis*), an important U.S. Eastern seaboard fish species, experimentally infected with *Mycobacterium marinum* and stimulated *M. marinum* antigen. No evidence of a DTH response was observed either grossly or histologically in our studies performed on Striped Bass. Pathological changes such as inflammation, myodegeneration, myoregeneration, hemorrhage, and granulomas were observed in both control and treatment groups histologically. Since an *in vivo* DTH assay requires multiple observations of a test subject, which may be impractical for fishes, we performed preliminary steps to lay the foundation for a non-lethal *in vitro* DTH assay similar to the QuantiFERON-TB Gold assay currently used in humans. This assay would quantify IFN-γ gene transcription of striped bass leukocytes in response to mycobacterial antigen exposure.

I devote my thesis work to my family Bruce, Margaret, and Marc Miller and my mentor Dr. David Gauthier. I owe my success to their indefatigable support and guidance. Thank you for teaching me how to eat an elephant… one bite at a time.

ACKNOWLEDGMENTS

My sincerest gratitude and appreciation to my committee members, Dr. David Gauthier, Dr. Dan Barshis, and Dr. Wolfgang Vogelbein for their counsel and assistance during this project. I would also like to sincerely thank my committee advisor, Dr. Gauthier, for giving me the opportunity to perform this project, and for his assistance, guidance, and most notably his unwavering patience.

A special thanks to Dr. Wolfgang Vogelbein for the use of his lab and equipment in the Virginia Institute of Marine Science (VIMS). I would also like to express my gratitude to Dr. Vogelbein's lab tech, Melanie T. Kolacy, for performing paraffin histology for this project, and for graciously teaching me how to perform it myself.

Thank you to my lab mate Amanda Pomposini and the RUMS students Austin Adkins, Jonathan Blubaugh, and Brandon Hamel for their assistance during this project.

TABLE OF CONTENTS

Page

LIST OF TABLES

Table Page

LIST OF FIGURES

Figure Page

I. INTRODUCTION

BACKGROUND

The T helper (Th) response of the vertebrate adaptive immune system can be subdivided into two general categories, Th1 and Th2, which predominate in reactions to intracellular and extracellular pathogens, respectively. Th2 cells promote B-cell proliferation for defense against parasites and other extracellular pathogens (Delves et al. 2011), while Th1 cells promote responses to intracellular bacterial and viral infections (Mosmann and Sad 1996; Savan et al. 2009). Th1 and Th2 type responses differ in the cytokine profiles that are produced (Delves et al. 2011). A hallmark of the Th1-type response is the secretion of interferon gamma (IFN-γ) (Mosmann and Sad 1996), a cytokine that can be traced back to an origin ~450 million years before the tetrapod-fish split (Mosmann and Sad 1996; Savan et al. 2009).

Interferon gamma is a dominant cytokine in vertebrate type IV hypersensitivity, also known as delayed type hypersensitivity (DTH). Of the four types of hypersensitivity (Type I, II, III, and IV), DTH is the only hypersensitivity that is cell rather than antibody-mediated (Delves et al. 2011). Type IV reactions in mammals are characterized by an erythema and induration that appears several hours after antigen exposure with the visible reaction reaching its maximum at 24-48 hours before subsiding (Delves et al. 2011). The exaggerated immune response of DTH has been used for the detection of disease, especially mycobacteriosis in humans. For instance, the widely used *in vivo* test, the tuberculin skin test (TST), is used to diagnose people who have been exposed to or who are infected with *Mycobacterium tuberculosis* or *Mycobacterium bovis*. Purified protein derivative (PPD) of *M. tuberculosis* is injected in subcutaneous tissue, and if an induration occurs at the injection in 48-72 hours post injection it is recorded (Huebner et al. 1993). If the tested individual possesses CD4+ T cells previously sensitized by exposure to *M. tuberculosis* or *M. bovis* antigen, these cells will release IFN-γ and other cytokines that will signal fluid release from the blood vessels (Cotran et al. 1999). Monocytes will then move from the blood vessel to the site of the antigen where the monocytes will be activated by the

presence of IFN-γ. Macrophages and leucocytes will then accumulate around the injected antigen, resulting in visible edema and induration.

Although TST has been used since the 1930s for *M. tuberculosis* screening, there is a high risk for cross reactivity to other *Mycobacterium* species (Huebner et al. 1993). People at risk for cross reactivity during a TST include those infected with *Mycobacterium avium* and those vaccinated with bacillus Calmette-Guérin, which is created using attenuated strains of *M. bovis* (Huebner et al. 1993; Diel et al. 2009; Slater et al. 2013; Manuel et al. 2007). Multiple exposures to TST can also act as a booster in individuals who have or have had a mycobacterial infection or were vaccinated with BCG (Huebner et al. 1993).

The QuantiFERON-TB Gold assay is a proposed replacement for the TST and is now being widely used for screening health care workers and people at risk for *M. tuberculosis* infections (TB) (Slater et al. 2013). Having equivalent sensitivity to the TST, the QuantiFERON-TB Gold assay has improved specificity in BCG vaccinated individuals and individuals with a non- *M. tuberculosis* mycobacterial infection (Slater et al. 2013). This assay is an IFN-γ release assay performed *in vitro* by exposing whole blood to an early secretory antigenic target-6 (ESAT-6) and culture filtrate protein-10 (CFP-10) that are encoded by genes located within the region of difference 1 which is present in all virulent laboratory and clinical strains of *M. tuberculosis* and *M. bovis* (Sørensen et al. 1995; Mahairas et al. 1996; Manuel et al. 2007; Soman et al. 2007; Pai et al. 2014). The exposed blood is then added to a plate coated with anti IFN-γ capture antibodies that will capture released IFN-γ from sensitized CD4+ T cells. A labeled detection antibody is then added to the plate where it will attach to antibodies with captured IFN-γ resulting in a color change. Because it uses ESAT-6 and CFP-10 proteins as stimulants, which are not present in *M. bovis* BCG, the QuantiFERON-TB Gold assay will not cross react with most NTM infections and BCG vaccinated individuals; unlike the TST (Farhat et al. 2006; Manuel et al. 2007; Connell et al. 2008; Diel et al. 2009; Slater et al. 2013; Pai et al. 2014).

Detection tests comparable to the TST have been performed in fishes to

determine if they produce a DTH response similar to mammals. Bartos and Sommer (1981) performed a DTH skin test in Rainbow Trout (*Oncorhynchus mykiss*) immunized with a 1:1 mixture of complete Freund's adjuvant, which contains inactivated *M. tuberculosis*, or phosphate buffered saline (PBS). Control fish were immunized with incomplete Freund's adjuvant (does not contain bacteria) or PBS (Bartos and Sommer 1981). Fish were then exposed to *M. tuberculosis* or *Mycobacterium salmoniphilum* "particulate". The fish were observed for induration at 24, 48 and 120 hours after exposure (Bartos and Sommer 1981). A response was best elicited in *M. tuberculosis* immunized fish 48 hours after being stimulated with an antigen (Bartos and Sommer 1981). There was a significantly greater DTH response in TB challenged fish that were exposed to *M. tuberculosis* particulate than the cross reactions of fish exposed to *M. salmoniphilum* particulate (Bartos and Sommer 1981). Soluble PPD produced no response in the fish, and it was necessary to challenge with particulate bacterial suspension to produce a DTH response (Bartos and Sommer 1981).

Other methods of testing DTH in fishes have also been performed in Sea Lampreys (*Petromyzon marinus*), Channel Catfish (*Ictalurus punctatus*) California Hagfish (*Eptatretus stoutii*), and Bowfin (*Amia calva*). The channel catfish did not exhibit a positive DTH response when tested with sonicated *Flavobacterium columnare* after previously being immunized with *F. columnare* and incubated for an unspecified duration (Pauley and Heartwell 1983). The authors noted that these results might be due to deviations from methods of previous studies such as the use of different sensitizing antigens that have not been known to produce DTH in mammals, or differences in the physiology of *I. punctatus* versus other species previously used (Pauley and Heartwell 1983). Also, *F. columnare* is an acute extracellular pathogen that is characterized by its adherence to the gills of host fish, and thus would not be expected to induce the same immune response as an intracellular pathogen such as *M. tuberculosis* or *M. avium* (Crowle et al. 1991; Gomes et al. 1999; Declercq et al. 2013). Finstad and Good injected Lamprey (*P. marinus*) intramuscularly with bovine gamma globulin (BGG) and complete Freund's adjuvant (Finstad and Good 1964). The

skin of *P. marinus* was tested with old tuberculin (O.T.) 21 and 30 days after the first immunization (Finstad and Good 1964). Of the stimulated *P. marinus*, about 70% that were tested with O.T. showed an induration and inflammation at the site of injection in 24 hours after being tested whereas all of the *P. marinus* not stimulated with complete Freund's adjuvant had negative reactions (Finstad and Good 1964). Papermaster et al. (1964) sensitized Hagfish (*E. stoutii*) by injecting the fish with BCG mixed with Freund's adjuvant intramuscularly and intracoelomically. The *E. stoutii* were then challenged with intradermal, intramuscular, and intracoelomic injections using old tuberculin (Papermaster et al. 1962; Papermaster et al. 1963; Papermaster et al. 1964). No response was observed to any of these secondary stimulations. Since *E. stoutii* is an evolutionarily early lineage, the black bullhead catfish (*Ameiurus melas*) was used as a "control" (Papermaster et al. 1962; Papermaster et al. 1963; Papermaster et al. 1964). It was noted that *A. melas* when stimulated with tuberculin would produce a DTH response (Papermaster et al. 1962; Papermaster et al. 1963; Papermaster et al. 1964), but details of the DTH response in *A. melas* were not provided. Bowfin (*A. calva*) were sensitized intramuscularly or intraperitoneally with *Ascaris lumbricoides* antigen in complete Freund's adjuvant then challenged with *Ascaris* antigen particulate 30 days later (Papermaster et al. 1964). Inflammation and induration were observed at the site of the injection within 72 hours (Papermaster et al. 1964). No reaction occurred in non-sensitized *A. calva* challenged with the *Ascaris* antigen (Papermaster et al. 1964). It is evident more extensive studies are needed of DTH in fishes to determine what fish are incapable of producing a DTH response. This will further the understanding of the evolution of the adaptive immune system and ultimately assist in the development of a non-lethal diagnostic assays for intracellular pathogens.

MYCOBACTERIOSIS ANALYSES IN STRIPED BASS

Currently there are no non-lethal diagnostic tests for mycobacterial infection or exposure in fishes. Detection relies on lethal sampling to collect

internal tissues for histology, bacteriological culture, and/or molecular tests (Gauthier and Rhodes 2009; Gauthier et al. 2011). Mycobacterial granulomas may be detected in histologic sections stained with hematoxylin and eosin (HE), and the Ziehl-Neelsen acid-fast stain may additionally be used to detect mycobacteria in granulomas (Prophet et al. 1992; Gauthier et al. 2008; Gauthier and Rhodes 2009). Visceral swabs or aseptically removed tissues may be cultured on a variety of selective liquid and agar media (Frerichs 1993; Teska et al. 1997; Chinabut 1999; Rhodes et al. 2004; Gauthier and Rhodes 2009). Once cultures are grown in broth and become turbid, DNA is extracted from cell pellets (Gauthier et al. 2011), PCR can then be performed using primers to amplify gene targets such as the 16S rRNA gene, 16S-23S internal transcribed spacer (ITS), heat-shock protein 65 (*hsp*65), exported repeated protein (*erp*), and the RNA polymerase B subunit (*rpo*B) (Kaattari et al. 2006; Gauthier and Rhodes 2009). The 16S rRNA gene is a favored target due to it being a highly conserved sequence in mycobacteria and the abundance of *Mycobacterium* spp. 16S gene sequences deposited in repositories such as GenBank and Ribosomal Differentiation of Microorganisms database (Gauthier and Rhodes 2009). Although well conserved, the 16S gene has been found to be restricted in its utility of differentiating closely related species of mycobacteria such as *Mycobacterium ulcerans* and *M. marinum*, which share a >99% overall nucleotide identity at this locus (Stinear et al. 2007; Gauthier and Rhodes 2009; Doig et al. 2012).

Striped Bass (*Morone saxatilis*) is an important commercial and recreational eastern seaboard fish species. The average U.S. commercial harvest from 2005-2014 & 2015 was 6.7 million pounds and over half of this was from the Chesapeake Bay (ASMFC 2016; Hoenig et al. 2017; NOAA 2017). Recreational fishing in the U.S. between 2005 and 2014 averaged 26.2 million pounds of Striped Bass annually (ASMFC 2016; Hoenig et al. 2017). In 1997, mycobacteriosis was first described in Chesapeake Bay Striped Bass and has been present in the population since (Baya 1998; Vogelbein et al. 1998; Rhodes et al. 2004; Matsche et al. 2010; Hoenig et al.; Groner 2018; Gauthier et al. 2008;

Hoenig et al. 2017). Surveys from 1998-1999, 2003-2005, 2005-2014, and unpublished data to 2016 have demonstrated that the prevalence of visceral and dermal disease associated mycobacteriosis in the Chesapeake Bay Striped Bass is greater than 50 % (Vogelbein et al. 1999; Cardinal 2001; Overton et al. 2003; Gauthier et al. 2008; Hoenig et al. 2017; Groner 2018). Fishery stock assessments showing an increase in natural, non-fishing mortality among Striped Bass in Maryland waters since 1999 suggest that mycobacteriosis is associated with increased natural mortality (Jiang et al. 2007). A model demonstrated diseased-associated mortality in Striped Bass from Chesapeake Bay (Gauthier et al. 2008). This model is based on assumptions such as irreversibility of the disease and age invariant disease-associated mortality (Gauthier et al. 2008). Although these assumptions are reasonable, they needed further verification in wild populations of Striped Bass. These concerns of the impact of mycobacteriosis on Striped Bass are now validated by recent findings which indicate an increase in the natural mortality rate of the Chesapeake Bay Striped Bass (Hoenig et al. 2017; Groner 2018). Understanding the DTH response in Striped Bass is the first step to assist in developing a non-lethal diagnostic assay to detect mycobacterial exposure in this species.

In this work, we perform an *in vivo* test similar to the TST, such as the one Bartos and Sommer performed in 1981, to expand our knowledge of DTH response in Striped Bass. We also describe first steps in development of a non-lethal *in vitro* test to detect mycobacteriosis in Striped Bass. This test will be similar to the QuantiFERON-TB Gold assay in that it will be performed *in vitro* with a blood sample, but differs in that it will measure transcription of IFN-γ in response to mycobacterial antigen stimulation, rather than directly measuring this cytokine by antibody capture. Using RT-qPCR to quantify the production of IFN-γ in fishes would only require a small portion of the IFN-γ gene from the species of interest to perform the analysis, rather than development of specific antibody.

Further characterizing DTH responses in fishes will assist in the development of non-lethal methods for detection of mycobacterial infections. Such methods would be useful to several stakeholders. Field researchers could

more effectively obtain data on disease status of fish populations, without needing to sacrifice fish. Aquaculturists and aquarists would benefit from non-lethal testing by being able to detect and remove a diseased individual within a stock or tank preventing the killing of an entire stock and loss in profit or the loss of highly valued individuals (Gauthier and Rhodes 2009). Identifying diseased individuals early and removing them from a small population could prevent future outbreaks of mycobacteriosis that commonly occur in controlled environments (Beran et al. 2006; Gauthier and Rhodes 2009).

II. METHODOLOGY

FISH MAINTENANCE

Striped Bass used in both the preliminary and primary studies were originally obtained from King and Queen State Fish Hatchery (Walkerton, VA) and maintained in a ~10000L recirculating system with trickle flow-through at ambient temperature and salinity tracking that of the York River in May of 2015 (Stevensville, VA). Photoperiod was matched to ambient conditions. All experimental procedures were performed in a BSL-2 isolation facility in recirculating 500L aquaria at the Virginia Institute of Marine Science (VIMS, Gloucester Point, VA). Individual tank systems were comprised of three 150L tanks connected to a sump and biofilter of approximately 50L capacity. Salinity was approximately 18 ppt, and temperature was maintained at 24°C. Alkalinity and pH were checked twice daily. Ammonia was monitored daily until levels had dropped consistently below 0.25 ppm and then checked biweekly. Alkalinity and pH were monitored regularly (3X/week) to maintain levels of 80-120 mg/mL, and >7.4, respectively. All laboratory procedures for the preliminary and primary studies were performed under an approved Virginia Institute of Marine Science (VIMS) Institutional Animal Care and Use Committee (IACUC) protocol (Project Number: IACUC-2016-02-17-10915-wrvoge).

M30-O1 PURIFIED PROTEIN DERIVATIVE (PPD)

Mycobacterium marinum purified protein derivative (PPD) was produced by modification of previous protocols (Wynne et al. 2012). *Mycobacterium marinum* M30-O1, a known pathogenic strain that has been used in previous studies (Gauthier et al. 2003; Gauthier et al. 2004), cultures were grown in Middlebrook 7H9 broth (BD, Franklin Lakes, NJ) with oleic albumin dextrose catalase (OADC) (Hardy Diagnostics, Santa Maria, CA) enrichment and 0.05% Tween-80 (Sigma, ST. Louis, MO) and incubated for two to three weeks at 30°C in a shaking incubator. Once cultures became turbid, 1 mL of the culture was added to a sterile 150 mL Erlenmeyer flask with 50 mL of room temperature

modified Watson and Reid Media prepared as previously described by Morrison (Morrison 1965). Cultures were placed back in the 30 °C shaking incubator to grow for another 2-3 weeks or until turbid. Cultures were inactivated via steam autoclave at 105 °C for 90 minutes. Inactivated cultures were then plated on Middlebrook 7H10 agar (BD, Franklin Lakes, NJ) plates with 10 % OADC enrichment and 0.05 % glycerol (Aldrich, Milwaukee, WI) to verify sterility then cooled overnight to 4 °C. Cooled inactivated cultures were transferred into a sterile 50 mL centrifuge tube and centrifuged in a Sorvall Legend RT+ centrifuge (Thermo Scientific, Waltham, MA) at 2000 × *g* for 5 min. Supernatant was separated into a new 50 mL centrifuge tube and pellets of particulate were frozen at -20 °C. Separated supernatant was then vacuum-filtered using 150 mL bottle top filter w/33mm neck with 0.22 µm cellulose acetate and low protein binding membrane (Corning Inc, Corning, NY) into a sterile 500 mL glass bottle. Collected liquid was transferred into 50 mL centrifuge tubes and centrifuged at 3000 × *g* for 10 minutes. The resulting supernatant was poured into a pre-weighed 50 mL falcon tube with 40 % (w/v) trichloroacetic acid (TCA) (Ameresco, Framingham, MA) solution in distilled water added to a final concentration of 4 %. Tubes were placed on a rocker for at least 30 minutes then placed in a dark room at room temperature for 15 to 18 hours allowing proteins to precipitate. Falcon tubes were then centrifuged at 2,600 × *g* for 15 minutes. Supernatant was carefully removed and the protein pellet was washed in 10 mL of 5 % NaCl (Aldrich, Milwaukee, WI) (w/v) + 0.5 % phenol (Bio Basic Canada INC., Markham, Ontario) (w/v) with a pH of 3.0 adjusted using 40 % (w/v) TCA solution then centrifuged at 2,600 × *g* for another 15 minutes. The wash was repeated three times or until the pH of the supernatant was between 2 and 3. The wet weight was measured before dissolving the washed pellets in 1.8 % (w/v) of $Na_2HPO_4\,7H_2O$ (Sigma, ST. Louis, MO) converted to $Na_2HPO_4\,2H_2O$ (pH 11) with the amount added equaling 2-2.5 mL per gram of wet weight. The dissolved pellet was then centrifuged at 2,600 × *g* for 10 minutes and then mixed with an equal volume of phosphate buffer 0.621 g of KH_2PO_4 (0.1 M) (Sigma, ST. Louis, MO) + 1.31 g $Na_2HPO_4\,7H_2O$ (0.1 M) containing 19.4 % (w/v) glucose (Sigma,

St. Louis, MO) and 0.5 % (w/v) phenol. The pH of the PPD was checked to be in the range of 6.7-6.9. A NanoDrop 2000c spectrophotometer (Thermo Scientific, Waltham, MA) was then used to determine the quantity and quality of the protein in the PPD preparation.

PRELIMINARY STUDY

Striped Bass (n = 18) were randomly distributed into individual tanks of systems designated for infected (2 systems, n = 12, maximum 3 fish per tank) or sham (HBSS)-injected (1 system, n = 6, maximum 2 fish per tank) fish. Fish were fed once per day with commercial feed (Ziegler Silver Pellets, 3 mm floating) and observed for abnormal behavior and or appearance. Mortalities were removed and examined for gross signs of mycobacteriosis (i.e. splenic granulomas).

Preliminary Fish Infection

Mycobacterium marinum strain M30 and *Mycobacterium marinum* M30-W12 (W12), a lab mutated attenuated strain from M30-O1, were grown in 25 mL of Middlebrook 7H9 broth with OADC enrichment and 0.05 % Tween-80 in a 30 °C Lab-Line® Orbit Environ-Shaker; (Lab-Line Instruments, Inc., Melrose Park, IL) for two weeks. Once cultures were turbid, they were placed in a 50 mL tube and centrifuged at 5000 × *g* for 30 minutes to form a pellet. The supernatant was discarded and the pellet was resuspended with Hank's Balanced Salt Solution (HBSS) (Corning Cellgro, Manassas, VA) and 0.05 % Tween 80 (VWR, Radnor, PA) filtered through a 0.2 µm cellulose acetate filter. The pellet was diluted to an optical density (OD) of 0.1 at 600 nm using a SmartSpec™ Plus Spectrometer (Bio-Rad, Hercules, CA). Dilutions and plate counts were performed in order to verify the number of bacteria injected. This was accomplished by performing tenfold serial dilutions that were plated in duplicate on Middlebrook 7H10 agar plates with 10 % OADC enrichment and 0.05 % glycerol. Culture plates were placed in a 30 °C Isotemp Incubator (Fisher Scientific, Hampton, NH) to grow for two weeks and then colonies were counted.

Fish were weighed (g), measured for fork length (mm), and tagged with BioMark Passive Integrated Transponder (PIT) tags (BioMark Diagnostics Inc, Richmond, Canada) at the time of injection. Six fish were then injected intracoelomically with 0.1 mL of filtered sterilized HBSS + 0.05 % Tween 80 (i.e., sham-injection) the remaining 12 were divided equally and injected with 0.1 mL HBSS + 0.05 % Tween-80 containing 1×10^7 cells/mL *M. marinum* M30 or with 0.1 mL HBSS + 0.05 % Tween-80 containing 1×10^7 cells/mL *M. marinum* W12.

Preliminary *In Vivo* DTH Assay

Eleven months post-injection, fish were randomly chosen from each treatment group (M30, W12, and sham) and injected subcutaneously in the caudal peduncle with either 0.1 mL sterile HBSS or 0.1 mL of heat-killed M30 (50 mg/mL) and 0.1 mL of heat-killed W12 (50 mg/mL) particulate. Heat-killed M30 was injected in the left side and heat-killed W12 was injected in the right side of the fish. Both injections were placed in the caudal peduncle at the posterior margin of the second dorsal fin above the lateral line. Fish exposed to HBSS were injected on the left side of the fish's caudal peduncle also at the posterior margin of the second dorsal fin above the lateral line. The exposed fish were then observed and the width of the caudal peduncle at the injection site was measured and observed 24, 48, and 72 hours post exposure.

All injections were administered with a 1 mL tuberculin syringe fitted with a 30-gauge needle. The width (mm) of the caudle peduncle at the injection sites were measured pre-exposure with Vernier calipers.

After the last caudal peduncle measurement fish were euthanized using MS-222 (Western Chemical Incorporated, Ferndale, WA) and macro pictures were taken of the injection sites using the following photography settings: TV mode, ISO 100 (Canon EOS 50D, United States). The flash settings used were mid-exposure 1/16, and E-TTL 0 (Canon Macro Ring Lite MR-14EX II). Spleens were removed and placed in Z-Fix zinc-buffered formalin (Anatech, Battle Creek, MI) for histological confirmation of mycobacterial infection. These spleens were

processed, observed, and scored for granuloma coverage as the spleens in the primary study.

PRIMARY STUDY

Striped Bass (n = 30, mean weight 526.33 g ± SD 186.62 and mean fork length = 357.17 mm ± SD 35.10) were randomly distributed into individual tanks of systems designated for infected (2 systems, n = 15, maximum 3 fish per tank) or sham (HBSS)-injected (2 systems, n = 15, maximum 3 fish per tank) fish. Husbandry conditions were as for the preliminary study.

Primary Fish Infection

M30 inoculum was prepared as previously described in the preliminary study and injected intracoelomically for the infected treatment (n = 15) and injected with HBSS for the shan trement (n = 15). Unfortunately, there was an error in which fish were initially injected with unfiltered (i.e. non-sterile) HBSS. This was noticed during plate dilutions of the original inoculum to verify the number of bacterial cells that were injected into challenged fish. Individual colonies from the contaminated plates were picked and placed in 20 µL of DEPC-treated water (GeneMate, Kaysville, UT) then disrupted at 100 °C for 5 minutes. PCR was performed using C1000 Thermal Cycler (Bio-Rad, Hercules, CA) in 15 µL final volume containing 1 µL of unknown disrupted bacteria DNA template. Each reaction contained 1 X Coral Load (QIAGEN, Valencia, CA), 1 X Buffer (BioPioneer, San Diego, CA) with 1.5 mM of $MgCl_2$, 0.2 mM dNTPs, 1 µM each of forward and reverse universal bacterial 16S primers (NU16S-F 5'-TCC TAC GGG AGG CAG CAG T-3'; NU16S-R 5'-GGA CTA CAGGGT ATC TAA TCC TGT T-3'), and 0.05 U Taq polymerase (Biopioneer, San Diego, CA). The thermocycler program was as follows: initial denaturation of 3 min at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, 55 °C annealing temperature for 45 s, and extension at 72 °C for 5 min, and a 7 min final extension at 72 °C.

Amplified DNA was electrophoresed on a 1.5 % agarose gel, stained with SYBR® Safe DNA Gel Stain (Invitrogen, Eugene, OR), and imaged with 360 nm

UV light. Amplified contaminates were then sequenced as described below with the initial primers for amplification. Sequenced data was then edited, aligned, and BLAST (Altschul et al. 1990) searches performed using Geneious R9 software (Drummond et al. 2010).

Fish were observed for two weeks for abnormal behavior or mortality, which did not occur. Sham-exposed fish were then re-inoculated with HBSS as described in the preliminary study and infected (challenged) fish were re-inoculated with 1×10^7 cells/mL *M. marinum* strain M30 grown and prepared as described in the preliminary study.

Primary *In Vivo* DTH Assay

Six and 12 weeks post-inoculation fish were randomly chosen from each treatment group (M30 and sham) and injected subcutaneously in the caudal peduncle and mid-venter region with either 0.1 mL sterile HBSS or 0.1 mL of heat-killed M30 (50 mg/mL) particulate and 0.1 mL of M30-O1 PPD (4.9 mg/mL). HBSS or heat-killed M30 were injected in the left side of the fish in the venter midway between the posterior margin of the pelvic fin and anterior root of the anal fin. Injections were performed in the caudal peduncle at the posterior margin of the second dorsal fin above the lateral line. PPD injections were given in the right side of the fish in the same locations, with the exception that right-side caudal peduncle injections were displaced caudally to facilitate measurement of indurations on both sides of the fish. The exposed fish were then observed and the width of the caudal peduncle at the injection site was measured and the venter sites observed 48, 96, and 168 hours post exposure.

All injections were administered with a 1 mL tuberculin syringe fitted with a 30-gauge needle. The width (mm) of the caudle peduncle at the injection sites were measured pre-exposure with Vernier calipers.

After the set duration of exposure fish were euthanized and macro photographs were taken of the injection sites as in the preliminary study. Fish were then exsanguinated via caudal venipuncture with a 25-gauge needle (BD, Franklin Lakes, NJ) fitted to a 3 mL syringe (BD, Franklin Lakes, NJ) with 0.1 mL

of sodium heparin (Sigma-Aldrich, St. Louis, MO) and HBSS solution (0.58 mg Na heparin/mL). Drawn blood was dispensed into heparinized Vacutainer tubes (BD, Franklin Lakes, NJ) and placed on ice. Cutaneous injection sites including all dermal layers and underlying muscle were removed and placed in Z-Fix zinc-buffered formalin for histology. Spleens were also removed and placed in Z-Fix zinc-buffered formalin as in the preliminary study for histological confirmation of mycobacterial infection. The anterior kidney then was aseptically removed and homogenized using a sterile glass TenBroek tissue homogenizer containing 1-3 mL of L-15 media (Sigma, St. Louis, MO) supplemented with 2 % fetal bovine serum (FBS, Intergen, Purchase, NY, U.S.A.), 100 U mL^{-1} penicillin, 100 µg mL^{-1} streptomycin, and 10 U mL^{-1} sodium heparin (L15/ 2 % FBS). The homogenized anterior kidneys were individually stored in a 15 mL Falcon tube with 3 mL of L15/ 2 % FBS and placed on ice.

The formalin-fixed spleen and injection sites were processed for paraffin histology according to Gauthier et al (2003). Sections of tissues were cut in 5 µm and stained with haematoxylin and eosin (H&E) (Gauthier et al. 2003) to be examined on an Olympus AX-70 light microscope. The spleens were graded in the order of 0-3 for the presence and areal coverage of granulomas which were distinguished by thick cellular layers surrounding a necrotic core. Spleens were scored based on the estimated percentage of granuloma coverage: Grade 1 – 1-10 %; Grade 2 – 10-50 %; Grade 3 – 50-100 %.

Skin samples were viewed for areas of abnormal tissue and scored from 0-3 for the pathological changes of inflammation, myodegeneration, and myoregeneration. Inflammation was characterized by the accumulation of leukocytes in an area. Myodegeneration was identified by the presence of myophagocytosis, cellular invasion of muscle fibers, and swollen, hyalinized and fragmented muscle fibers. Myoregeneration was identified via the presences of myoblasts, striated acidophilic cells containing actomyosin bundles. Only abnormal (pathological) areas were scored in order to prevent plane of section bias. For instance, depending on if a sample was cut vertically or horizontally from the muscle's natural direction or how much of the injection site (needle track

and area of diffusion for injected material) was present in the section could lead to misinterpretation of pathological changes. If a sample was graded as a 0 then there were no pathological changes observed. For samples with pathological change, scoring was based on the estimated percentage of altered area comprised of the given specific histopathological finding: Grade 1 – 1-10 %; Grade 2 – 10-50 %; Grade 3 – 50-100 %. The presence and absence of granulomas and hemorrhaging, which was identified by an accumulation of uncontained erythrocytes, were also recorded in the skin samples. All histology (preliminary and primary) was viewed using a double blind method to prevent biased scoring.

CELL CULTURE

Extracted anterior kidney cells and blood samples (only from the primary study) were placed in culture following previous published protocols (Gauthier et al. 2003). Blood samples were first centrifuged at 500 × *g* for 10 minutes. Once separated, the buffy coat was removed and placed in a 15 mL Falcon tube with 3 mL of L-15/ 2 % FBS and leucocytes processed as for the anterior kidney. Homogenized anterior kidneys cells and peripheral blood lymphocytes (PBL) in 3 mL of L-15/ 2 % FBS were pelleted by centrifugation at 4 ˚C for 10 minutes at 500 × *g* and washed three times via suspension in L-15/ 2 % FBS. After the third wash, the pellets were re-suspended in 5 mL of L-15/ 2 % FBS and layered on an equal volume of 32 % (AK) or 46 % (PBL) Percoll™(GE Healthcare, Little Chalfont, United Kingdom) in HBSS without phenol red, then centrifuged at 4 ˚C for 40 minutes at 500× *g*. Isolated leucocytes were removed from the media/Percoll™ interface; pelleted and then washed as previously described and suspended in L-15/ 2 % FBS for counting. The number of viable leucocytes isolated from each fish was determined by trypan blue exclusion (0.1 % of trypan blue solution (Sigma-Aldrich, St. Louis, MO) in L-15/ 2 % FBS). Leucocytes were suspended in L-15/ 2 % FBS at 2×10^7 viable cells mg/mL and dispensed at 50 µL per well (1×10^6 cells) in tissue culture treated 96 well flat-bottom plates (Costar, Corning, NY). Fifty µL per well of L-15 supplemented with 5 % FBS, 100 U mL^{-1}

penicillin and 100 µg mL^{-1} streptomycin (L-15/ 5 % FBS) was then added per well. Plates were incubated overnight at 20 °C in a low temperature illuminated incubator (818 Thermo Electron Corporation, Waltham, MA).

When suspending the six week leucocytes in L-15/ 2 % FBS, there was a miscalculation in the number of viable cells mg/mL that were plated. Instead of dispensing 1×10^6 viable cells mg/mL, 1×10^4 viable cells mg/mL were plated.

CHARACTERIZATION OF THE STRIPED BASS IFN-γ GENE

The Striped Bass IFN-γ mRNA was extracted from deposited transcriptomic data (Li et al. 2014) using BLAST queries of annotated IFN-γ genes from other fishes. The "best hits" from the BLAST search were then *de novo* assembled and manually curated using Geneious R9 (Biomatters; Drummond et al. 2010). The resulting Striped Bass IFN-γ exons were then pairwise aligned and exon boundaries predicted, to well-annotated IFN-γ genes of European Seabass (*Dicentrarchus labrax*: KJ818329); Atlantic Halibut (*Hippoglossus hippoglossus*: GU985450); and Rainbow Trout (*Oncorhynchus mykiss*: FJ184375.1). The protein structure of the Striped Bass IFN-γ gene was predicted using Phyre2 (Kelley et al. 2015).

The striped bass IFN-γ gene including introns and untranslated regions (UTRs) was characterized using IFN-γ exon sequences as a starting point. Based on the INF-γ exon sequence, the Striped Bass IFN-γ mRNA was pairwise aligned to the Atlantic Halibut's well-annotated IFN-γ gene in order to create sequencing primers. Primers were optimized by PCR gradient in a C1000 Thermal Cycler in 15 µL final volume containing 1 µL of extracted Striped Bass DNA template. Each reaction contained 1 X Coral Load (QIAGEN, Valencia, CA), 1 X Q-solution (QIAGEN, Valencia, CA), 0.2 mM dNTPs, 1 µM each of forward and reverse primer, 1 PCR buffer containing 1.5 mM $MgCl_2$ (QIAGEN, Valencia, CA), and 0.05 U Taq polymerase (Top-Taq, QIAGEN, Valencia, CA). PCR was also performed with BioPioneer Taq as described above. The thermocycler program was as follows: initial denaturation of 3 min at 95 °C; 35 cycles of denaturation at 95 °C for 30 s, the corresponding annealing

temperature for 45 s, and extension at 72 °C for 5 min, and a 7 min final extension at 72 °C. Amplified DNA was electrophoresed on a 1.5 % agarose gel, stained with SYBR® Safe DNA Gel Stain, and imaged with 360 nm UV light.

TABLE 1. Striped Bass IFN-γ primer pairs. These primers were developed for amplification of portions of the Striped Bass IFN-γ gene.

Primer Set Name	Forward and Reverse Primers	5' to 3' Sequence	Product length of cDNA amplicon (bp)	Annealing Temperature
Primers_1	A_utr_exon1_146_F B_utr_exon1_190_R	5' AAACCCAAGCAGGTCACACC 3' 5' GAGCTCCTGACCTGACACAC 3'	146	65 °C
Primers_2	B_exon4-utr_105_F B_exon4-utr_105_R	5' CGAGCTCGCTGTCTGATGAG 3' 5' TGTCGTTGTGCCTGTTTGTG 3'	105	65 °C
Primers_3	A_exon3-4_127_F A_exon3-4_127_R	5' TGAAGGAACTGCAGGCTCTC 3' 5' CGCCTCCTCTGCATCTCATC 3'	127	65 °C
Primers_4	F_1_exon3-4_271_F F_1_exon3-4_271_R	5' TGTTTATGGGCGGCGTTTTG 3' 5' TGGACGACGCGTTTATCCAT 3'	271	65 °C
Primers_5	B_exon2-3_67_F B_exon2-3_67_R	5' GGAAACCCGTCTTCTCCAGG 3' 5' CCGCCCATAAACAGCCTCTT 3'	67	64 °C
Primers_7	A_utr_exon1_146_F C_exon1-2_172_R	5' AAACCCAAGCAGGTCACACC 3' 5' CAGCTGCTTCAACATCTCGC 3'	146	61 °C
Primers_11	F_1_exon3-4_271_F A_exon3-4_127_R	5' TGTTTATGGGCGGCGTTTTG 3' 5' CGCCTCCTCTGCATCTCATC 3'	271	60 °C

SEQUENCING

PCR products with predicted product lengths were purified with Exo-SAP-IT (Thermo Scientific, Waltham, MA) according to the manufacturer's directions. Direct sequencing was performed on PCR products bi-directionally using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City,

CA) and the primers used for initial amplification. Sequencing reactions were run in duplicates to generate a total of four forward and reverse sequences.

CLONING

Large PCR products (1,000 base pairs or more) were cloned using a TOPO TA Cloning® Kit for Sequencing (Invitrogen, Eugene, OR) according to the manufacturer's directions, then sequenced.

SEQUENCE ANALYSIS

Sequenced data was edited, aligned, and BLAST searches performed using Geneious R9 software (Drummond et al. 2010) in order to confirm that the target sequence was amplified. Consensus sequences were generated from at least four total forward and reverse sequences.

From the primers listed above 3,000 base pairs (bp) of the estimated 5,000 bp of the Striped Bass IFN-γ gene were sequenced and characterized. To further assist in characterizing the IFN-γ gene, sequence data was referenced aligned using Geneious R9 software to European Seabass (*Dicentrarchus labrax*: KJ818329) (Ortiz et al. 2014) due to its high nucleotide and amino acid pairwise identity to the Striped Bass mRNA IFN-γ . Collected sequence data was later successfully mapped to the newly available Striped Bass (*Morone saxatilis*: JTCL00000000.1) (Reading et al. 2016). This alignment of sequence data and known exons remained syntenic with previously generated assemblies. Putative elements such as the TATA box; internal ribosome entry site (IRES); polyadenylation sites; and cytoplasmic polyadenylation elements (CPE) found in the 5' and 3' untranslated regions (UTRs) were used to identified the Striped Bass UTRs. The nucleotide sequence (Table 2) of the elements were then aligned to a large excised segment of the annotated Striped Bass gene and the length of the UTRs were predicted where the TATA box began and the CPE and polyadenylation sites ended (Pesole et al. 2001; Furnes et al. 2009; Ivshina et al. 2014).

TABLE 2. Putative element sequence of the 5' and 3' untranslated regions (UTRs). These elements were used to identify the Striped Bass UTRs.

Putative Elements	UTR Found In	Sequence	Number of Elements
TATA box [1]	5'	TATATA	1
Internal Ribosome Entry Site (IRES) [1]	5'	TGATTTAAAGG	1
Polyadenylation Sites [2]	3'	TTATTT	10
Cytoplasmic Polyadenylation Elements (CPE) [3]	3'	TTTTAT	4

[1] Furnes et al. 2009
[2] Pesole et al. 2001
[3] Ivshina et al. 2014

IN VITRO DTH ASSAY

Cells were incubated overnight and then exposed in triplicate to 100 µL of high (1 mg/mL) (PPD-H) and low (0.5 mg/mL) (PPD-L) concentrations of M30-O1 PPD in L-15/ 5 % FBS for a final concentration of 0.5 and 0.25 mg/mL. Cells were also exposed to 50 mg/mL heat-killed M30-01 in L-15/ 5 % FBS. Control cells received L-15/ 5 % FBS. Exposed cells were then incubated for 48 or 72 hours.

After incubation, plates were centrifuged at 500 × *g* for 10 minutes. The supernatant was carefully removed from each well and replaced with 250 µL of TRIzol® Reagent (Ambion, Carlsbad, CA) while pipetting slowly up and down to suspend and disrupt the cells. Contents of each well were removed and place in a labeled 1.7 mL microcentrifuge tube and stored at -80 ˚C until RNA extraction.

RNA EXTRACTIONS

Frozen exposed cells in TRIzol® Reagent were thawed to room temperature then RNA was extracted according to the manufacturer's instructions. RNA-grade glycogen (Thermo Scientific, Waltham, MA) was used at 5 µg per reaction as suggested by the TRIzol® Reagent manufacturer's instructions during the RNA precipitation step. The resulting isolated RNA pellet was resuspended in 10 µL of RNase-free water then treated using RNase-free DNase I (Thermo Scientific, Waltham, MA) according to the manufacturer's instructions. The total RNA concentration of each reaction was measured using the Qubit 2.0 Fluorometer (Thermo Scientific, Waltham, MA) then stored at -80 ˚C for later use.

REVERSE TRANSCRIPTASE –QUANTITATIVE REAL TIME PCR (RT-qPCR)

RT-qPCR was performed for the Striped Bass IFN-γ gene and two housekeeping genes: ribosomal protein L9 (L9), and elongation factor-1 (EF-1α) (Geist et al. 2007; Jeon et al. 2011). These housekeeping genes were chosen due to their consistency and stability in RT-qPCR after immunological stimulation in Striped Bass and European Seabass (Geist et al. 2007; Jeon et al. 2011). The efficiencies for the housekeeping genes was established via making three tenfold dilutions of an extracted RNA sample from the liver of a sham-exposed Striped Bass in the preliminary study. RT-qPCR was performed as stated below with the corresponding annealing temperature for the primers being tested (Table 3). The efficiencies of the housekeeping primers were determined using Bio-Rad CFX Manager™ Software.

The Striped Bass IFN-γ (SBIFN) primers were used to detect the production of IFN-γ post the *in vitro* DTH assay. Efficiency for the SBIFN primers were performed as above with RNA extracted from a collected Striped Bass anterior kidney. However due to consistently high Cq values produced (~ 32 Cq) using SBIFN primers a sample from the original efficiency post amplification (with undiluted RNA) was Exo-SAP-IT according to the manufacturer's directions. The

sample was then diluted to 10^{-8}-10^{-11}. The efficiencies of the SBIFN primers were also determined using Bio-Rad CFX Manager™ Software.

TABLE 3. RT-qPCR target gene primer sets.

Target Primers	Forward and Reverse Primers	5' to 3' Sequence	Annealing Temperature
EF-1α [1]	EF-1α-F EF-1α-R	5'-CTT GAC GGA CAC GTT CTT GA-3' 5'-GTG GAG ACC GGT GTC CTG AA-3'	58 ˚C
L9 [2]	L9- F L9-R	5'-AAG CTT CGT GTG GAT AAA TGG TG-3' 5'-GAC GAT GGT GCG GAC TGT G-3'	59 ˚C
Striped Bass IFN-γ (SBIFN)	1930F 2124R	5'-CAA AAC GCC GCC CAT AAA CA-3' 5'-GTG TGT CAG GTC AGG AGC TC -3'	64 ˚C

[1] Jeon et al. 2011
[2] Geist et al. 2007

RT-qPCR was performed using a Bio Rad CFX96™ Real-Time System C1000™ Thermal Cycler and iTaq™ Universal SYBR® Green 1-Step Kit (Bio-Rad, Hercules, CA). Extracted DNase-treated RNA was not normalized because the nucleic acid content of each sample was found to be less than 20 ng/mL. Two µL of the sample was added to a 1 x concentration of iTaq™ Universal SYBR® Green 1-Step Reaction Mix; 1 x concentration of iScript™ Reverse Transcriptase; 300nM each of forward and reverse primers; DEPC-treated water was added to bring the total volume to 15 µL. The cycling conditions were as follows: 10 min at 50 ˚C with a 1 min denaturation at 95 ˚C; then 40 cycles of 10 s at 95 ˚C and 30 s at the proper annealing temperature for the selected primer set. A melt curve was performed ranging from 65 ˚C to 95 ˚C in 0.5 ˚C increments.

Anterior kidney (AK) and PBL exposed cells from three sham injected fish skin tested with HBSS and three challenge injected fish skin test with M30 PPD and heat-killed M30 were selected for the first run of RT-qPCR (Figure 1). From the PBL cells triplicates of control cells (cells exposed to L15/ 5 %) and PPD-H cells (cells exposed to high PPD) were tested with the housekeeping primers and Striped Bass IFN-γ (SBIFN). Triplicates of AK control cells and PPD-H cells were tested with SBIFN.

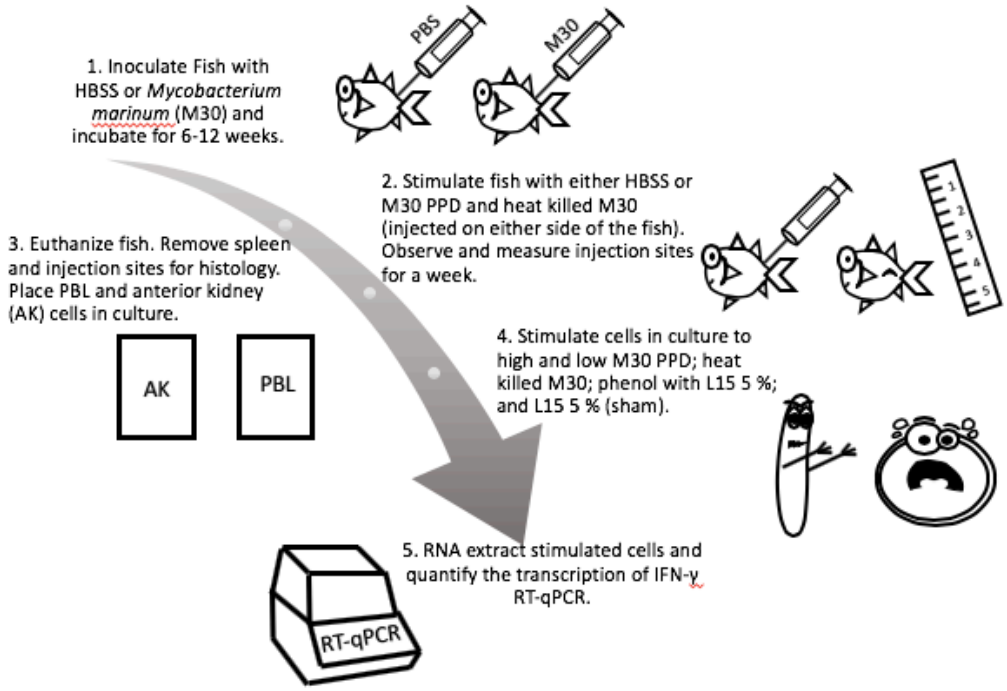

FIGURE 1. Overview of primary *in vivo* and *in vitro* DTH assay in Striped Bass.

STATISTICAL ANALYSES

All statistical analyses were performed in RStudio, Version 1.1.383 - © 2009-2017 RStudio, Inc., except for the Dunnett's *post hoc* test which was

performed by hand (Dunnett 1955). A Single Factor Repeated Measures ANOVA was performed using the packages “lme4” and “lemerTest” according to the model: lmer(Measurement ~ Hour + (1 | ID), data). Kruskal-Wallis Rank Test was performed using the package “stats” according to the model: kruskal.test(Pathology ~ Treatment, data) (Kruskal and Wallis 1952). The Dunn’s *post hoc* test was performed using the package “FSA” according to the model: dunnTest(Pathology ~ Treatment, data, method = “bonferroni”) (Dunn 1964). The Fisher’s Exact Test was performed using the package “stats” according to the model: fisher.test(Table, con.int = T, conf.level = 0.95) (Upton 1992).

III. RESULTS

IN VIVO DTH ASSAY IN STRIPED BASS

During the *in vivo* DTH assays no indurations or swelling were seen and only minor hemorrhage at the injection site was observed (Figure 2) in both the preliminary and primary study. Observations of hemorrhage were anecdotally noted in the preliminary study but recorded as data in the primary study. In the primary study the most scale pocket hemorrhage was observed in challenged fish (fish intracelomically injected with live *M. marinum*) six weeks post-injection stimulated with heat-killed M30 at both caudal and venter sites, 168 hours post exposure. No hemorrhage was observed in challenged fish six weeks post-injection stimulated with HBSS or M30 PPD at either the caudal peduncle or the venter. Hemorrhage was also observed more frequently in the venters of sham-exposed fish (fish intracelomically injected with HBSS) six weeks post-injection stimulated with heat-killed M30 at 96 and 168 hours (Table 4).

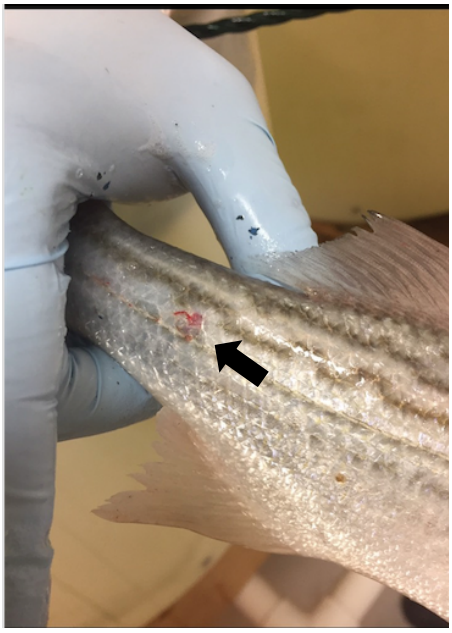

FIGURE 2. Scale pocket hemorrhage (arrow) at the caudal peduncle.

Unlike fish six weeks post-injection, at 12 weeks post-injection hemorrhage occurred less frequently in sham-exposed and challenged fish post stimulation in all treatment groups (Table 4). When hemorrhage was observed in any treatment of fish 12 weeks post-injection it was mainly seen in the venter injection sites. The most hemorrhage observed in fish 12 weeks post-injection was in sham-exposed fish stimulated with heat-killed M30 at the venter site (Table 4). No hemorrhage was observed in sham-exposed and challenged fish stimulated with HBSS in the caudal peduncle at 12 weeks post-injection (Table 4).

TABLE 4. Percentage of scale pocket hemorrhage in the primary study. Hemorrhage was observed in sham-exposed and challenged fish in the caudal peduncle and or venter injection sites of fish.

Scale Pocket Hemorrhage Post Stimulation						
Injection	**Treatment**	**n 6wk/12wk**	**Tissue**	**Post 48 hr. 6wk/12wk**	**Post 96 hr. 6wk/12wk**	**Post 168 hr. 6wk/12wk**
HBSS	HBSS	3/ 3	Caudal	0 %/ 0 %	0 %/ 0 %	33.33 %/ 0 %
HBSS	HBSS	3/ 3	Venter	0 %/ 33.33 %	0 %/ 33.33 %	33.33 %/ 0 %
HBSS	Heat-Killed M30	4/ 3	Caudal	0 %/ 33.33 %	25 %/ 0 %	0 %/ 0 %
HBSS	Heat-Killed M30	4/ 3	Venter	0 %/ 66.66 %	50 %/ 66.66 %	75 %/ 33.33 %
HBSS	M30 PPD	4/ 3	Caudal	0 %/ 33.33 %	0 %/ 0 %	0 %/ 0 %
HBSS	M30 PPD	4/ 3	Venter	0 %/ 33.33 %	0 %/ 0 %	25 %/ 0 %
Live M30	HBSS	3/ 3	Caudal	0 %/ 0 %	0 %/ 0 %	0 %/ 0 %
Live M30	HBSS	3/ 3	Venter	0 %/ 33.33 %	0 %/ 0 %	0 %/ 0 %
Live M30	Heat-Killed M30	4/ 2	Caudal	0 %/ 50 %	50 %/ 0 %	100 %/ 50 %
Live M30	Heat-Killed M30	4/ 2	Venter	50 %/ 0 %	50 %/ 50 %	100 %/ 50 %
Live M30	M30 PPD	4/ 2	Caudal	0 %/ 50 %	0 %/ 0 %	0 %/ 0 %
Live M30	M30 PPD	4/ 2	Venter	0 %/ 50 %	0 %/ 0 %	0 %/ 0 %

There was no difference in the caudal width within treatment groups in the preliminary *in vivo* DTH study (Single Factor Repeated Measures ANOVA for every treatment group: $p > 0.05$) (Figure 3).

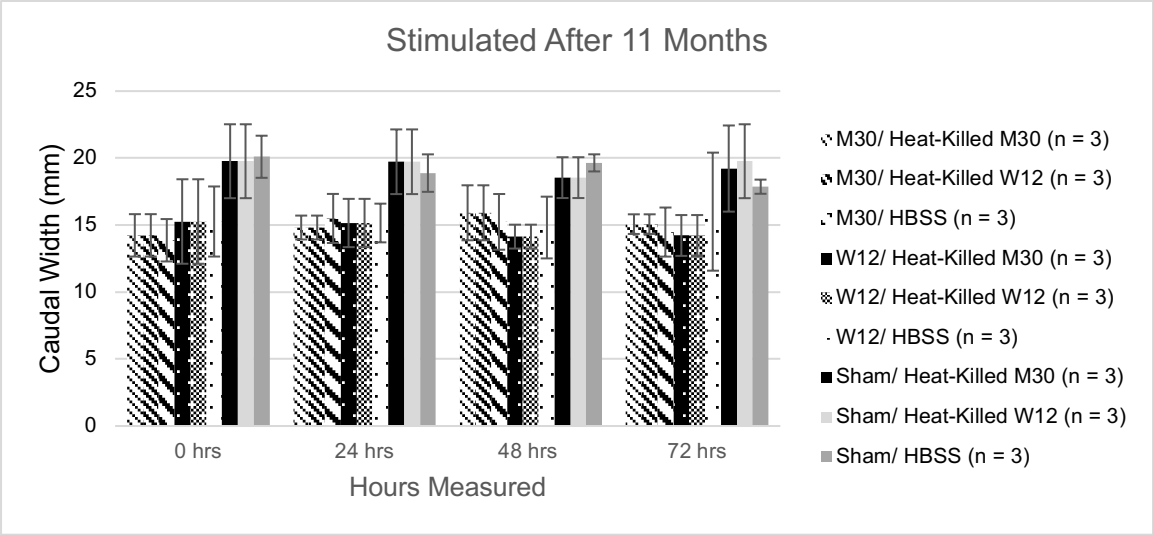

FIGURE 3. Preliminary fish stimulated 11 months post-infection. Mean (±SD) caudal width (mm) of fish in preliminary study after stimulation with heat-killed *Mycobacterium marinum* M30 (heat-killed M30) and heat-killed *M. marinum* W12 (heat-killed W12) or HBSS 11 months post-inoculation with HBSS (sham; solid bars), *M. marinum* M30 (M30; diagonal striped bars), or *M. marinum* W12 (W12; dotted bars). No significant differences were found in caudal peduncle width over time in any treatment group (Single Factor Repeated Measures ANOVA, $p > 0.05$).

In the primary study, the caudal width of sham-exposed fish stimulated with HBSS six weeks post-injection was greater at 168 hours than before stimulation (time point 0) (Single Factor Repeated Measures ANOVA: n = 3, df= 2, p = 0.0294) with Dunnett's *post hoc* test (Dunnett 1955) (Figure 4). There was no difference in caudal peduncle width of sham-exposed fish stimulated with M30 PPD six weeks post-injection (Single Factor Repeated Measures ANOVA: n = 4,

df = 3, p = 0.327) and heat-killed M30 (Single Factor Repeated Measures ANOVA: n = 4, df = 3, p = 0.499). The caudal width of challenged fish stimulated with HBSS six weeks post-injection was significantly different over time (Single Factor Repeated Measures ANOVA: n = 3, df = 2, p = 0.0494). However statistical significance between time points was indistinguishable via the Dunnett's test, likely due to the small sample size. There was no difference over time in the caudal width of six week challenged fish stimulated with M30 PPD (Single Factor Repeated Measures ANOVA: n = 4, df = 3, p = 0.741) and heat-killed M30 (Single Factor Repeated Measures ANOVA: n = 4, df = 3, p = 0.35).

At 12 weeks post-injection, no stimulants produced an increase in caudal width in any treatment group [Single Factor Repeated Measures ANOVA: (M30 PPD) n = 3,df = 2, p = 0.849; (heat-killed M30) n = 3, df = 2, p = 0.794; (HBSS) n = 3, df = 2, p = 0.445] (Figure 5). A significant increase was observed in caudal width of fish exposed to M30 for 12 weeks and stimulated with M30 PPD (Single Factor Repeated Measures ANOVA: n = 2, df = 1, p = 0.0374), but significance could not be determined between time points due to the small sample size. Fish exposed to M30 for twelve weeks and stimulated with HBSS or M30 PPD showed no difference in caudal width over time [Single Factor Repeated Measures ANOVA: (HBSS) n = 3, df = 2, p = 0.775; (heat-killed M30) n = 2, df = 1, p = 0.91].

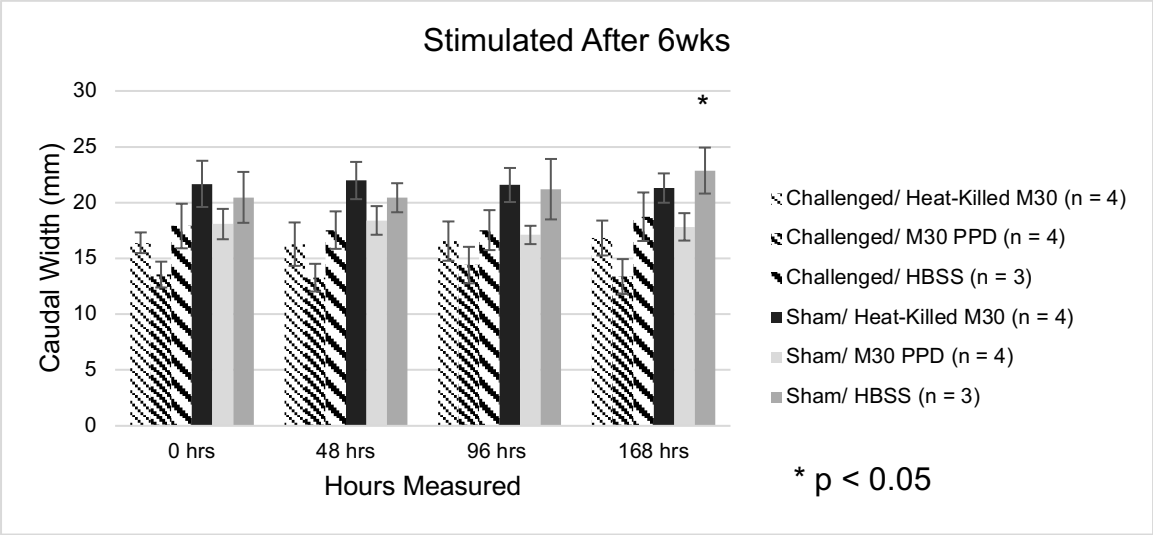

FIGURE 4. Challenged and sham-exposed fish stimulated six weeks post-infection. Mean (±SD) caudal width (mm) of fish in primary study measured over time after being stimulated with *M. marinum* M30 PPD (PPD), heat-killed *M. marinum* M30 or HBSS six weeks post-inoculation with HBSS (Sham) (solid bars) or *M. marinum* M30 (diagonal striped bars).

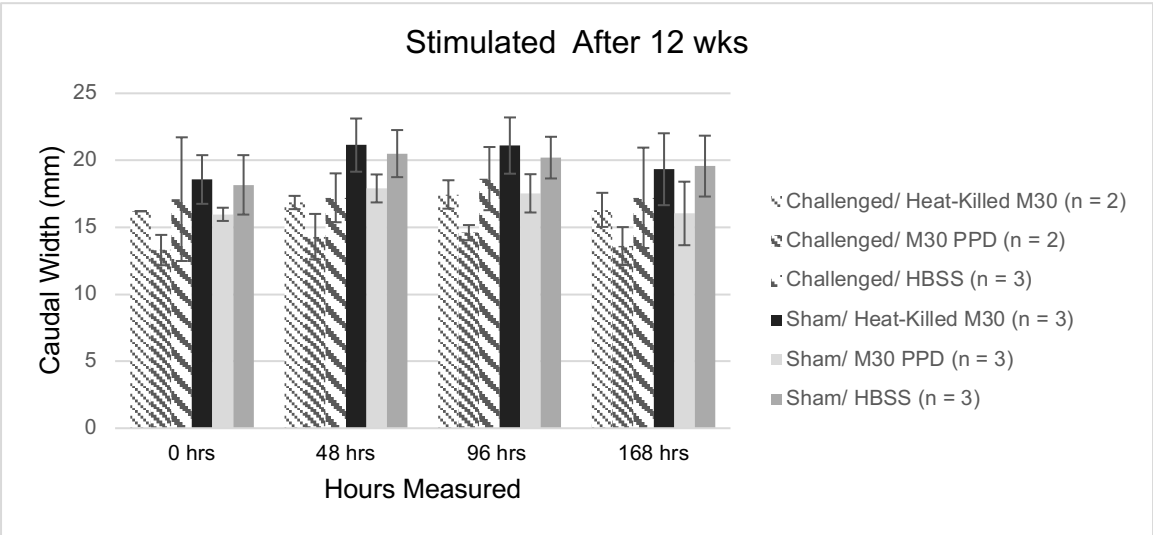

FIGURE 5. Challenged and sham-exposed fish stimulated 12 weeks post infection. Mean (±SD) caudal width (mm) of primary fish measured over time after being stimulated with *M. marinum* M30 PPD (PPD), heat-killed *M. marinum* M30 or HBSS 12 weeks post-inoculation with HBSS (Sham) (solid bars) or *M. marinum* M30 (diagonal striped bars).

HISTOLOGY

Spleens from M30-injected fish in both the preliminary and primary study possessed granulomas similar to that of challenged spleens in previous studies (Figure 6) (Gauthier et al. 2003). Granuloma severity was greater in fish inoculated with *M. marinum* M30 vs. fish inoculated with either HBSS or *M. marinum* W12 (Kruskal-Wallis Rank Test; χ^2 = 25.473, df = 4, p = 4.0e-05, Dunn's *post hoc* test) (Figure 7).

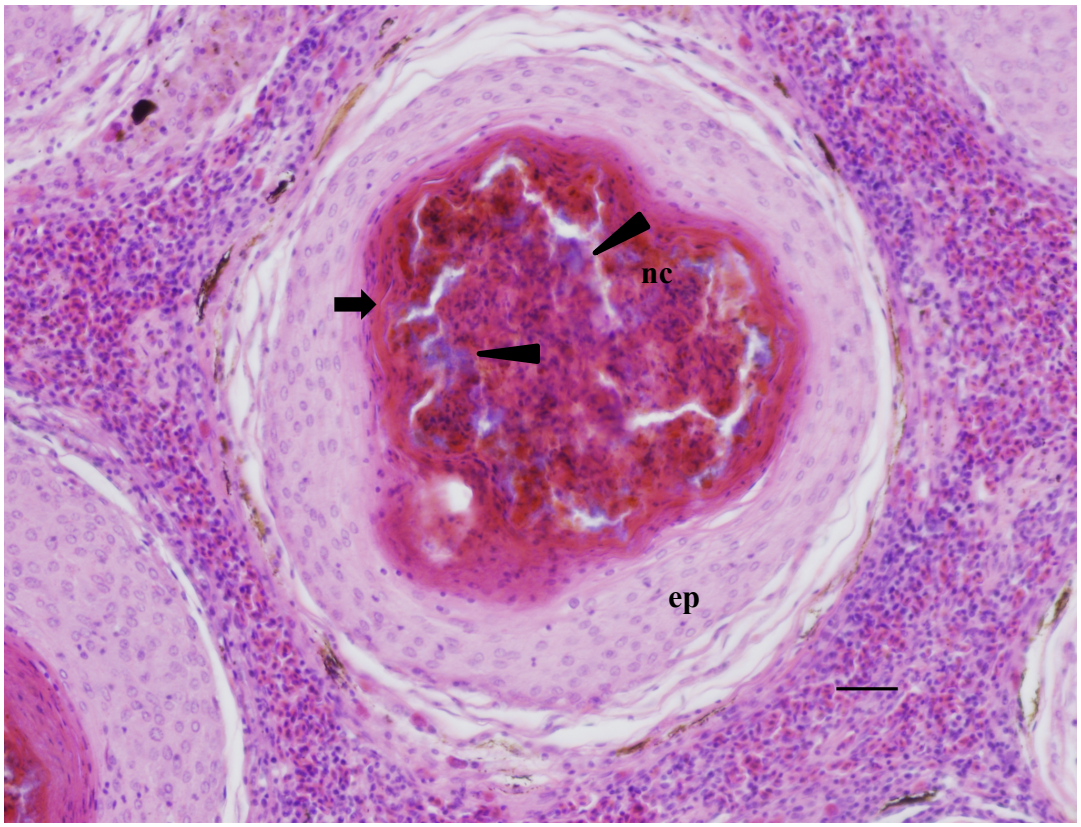

FIGURE 6. A granuloma in the spleen of a Striped Bass. This fish was inoculated with *Mycobacterium marinum* M30. An epithelioid cell layer (ep) and a compressed epithelioid cell layer (arrow) surround a necrotic core (nc). Bacteria (arrowheads) are present in the necrotic core. Hematoxylin and eosin stain (bar = 50 µm).

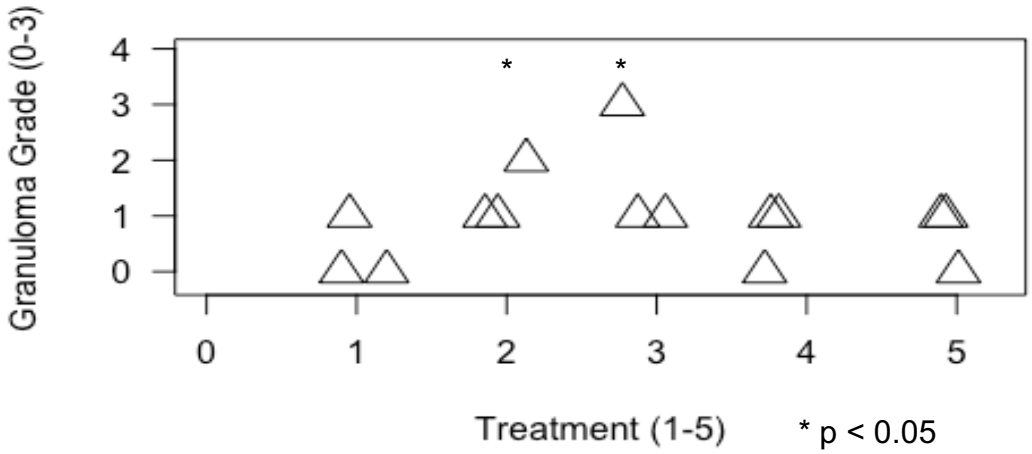

FIGURE 7. Granuloma severity in spleens of preliminary fish. These fish were inoculated with HBSS, *M. marinum* M30, or *M. marinum* W12. The granuloma grade is based on the estimated percentage of granuloma areal coverage: Grade 1 – 1-10 %; Grade 2 – 10-50 %; Grade 3 – 50-100 %. Treatments are: (1) sham fish stimulated with HBSS; (2) M30 challenged fish stimulated with HBSS; (3) M30 challenged fish stimulated with heat-killed M30 and heat-killed W12; (4) W12 challenged fish stimulated with HBSS; and (5) W12 challenged fish stimulated heat-killed M30 and heat-killed W12.

In the primary study of fish stimulated 6 and 12 weeks post-infection granuloma severity was greater in spleens of fish challenged with *M. marinum* M30 than spleens of sham-exposed fish (Kruskal-Wallis Rank Test: 6wk, χ^2 = 61.996, df = 3, $p < 0.01$; 12wk, χ^2 = 42.994, df = 3, $p < 0.01$; Dunn's *post hoc* test) (Figure 8).

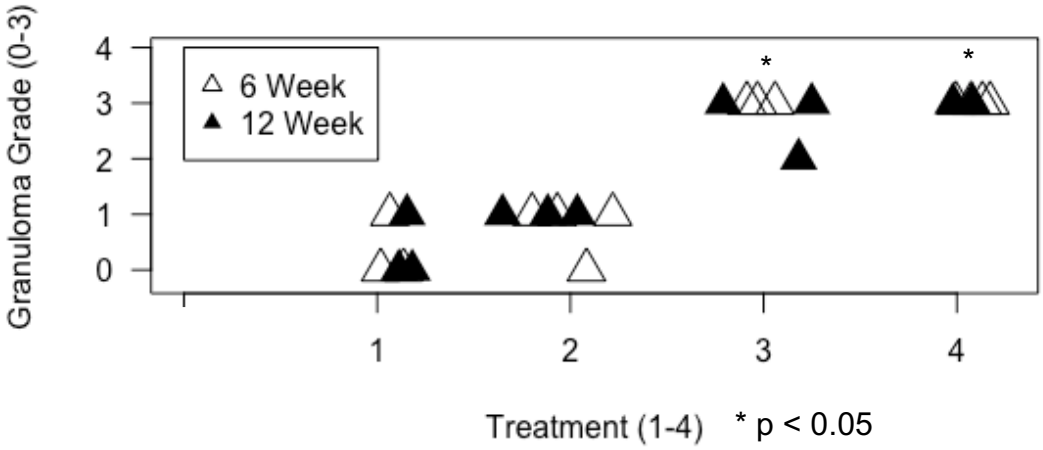

FIGURE 8. Granuloma severity in spleens of primary fish. Granuloma coverage graded (0-3) in spleens of sham-exposed & challenged fish post 6 (open triangles) and 12 weeks (closed triangles) from injection. Scale of granuloma coverage is the same as stated in Figure 7. Treatments: Treatments: (1) sham-exposed fish stimulated with HBSS; (2) sham-exposed fish stimulated with heat-killed M30 and M30 PPD; (3) challenged fish stimulated with HBSS; (4) challenged fish stimulated with heat-killed M30 and M30 PPD.

Inflammation

In histological samples of fish collected a week post-stimulation with heat-killed M30, *M. marinum* particulate was visible in both the caudal peduncle and venter injection sites (Figure 9). Qualitatively more inflammation was observed in M30 heat-killed and M30 PPD-stimulated fish than in those stimulated with HBSS (Figure 10). There was a difference between sham-exposed and challenged fish stimulated in the venter with M30 PPD 12 weeks post-infection (Kruskal-Wallis Rank Test: df = 1, p = 0.0455). There were no differences between sham-exposed fish and challenged fish when stimulated with HBSS or heat-killed M30 in the caudal peduncle or venter 6 and 12 weeks post-infection [Kruskal-Wallis Rank Test (6wk & 12wk, caudal peduncle): All treatment comparisons, df = 1, p > 0.05; (6wk, venter): HBSS stimulated and heat-killed M30, df = 1, p > 0.05].

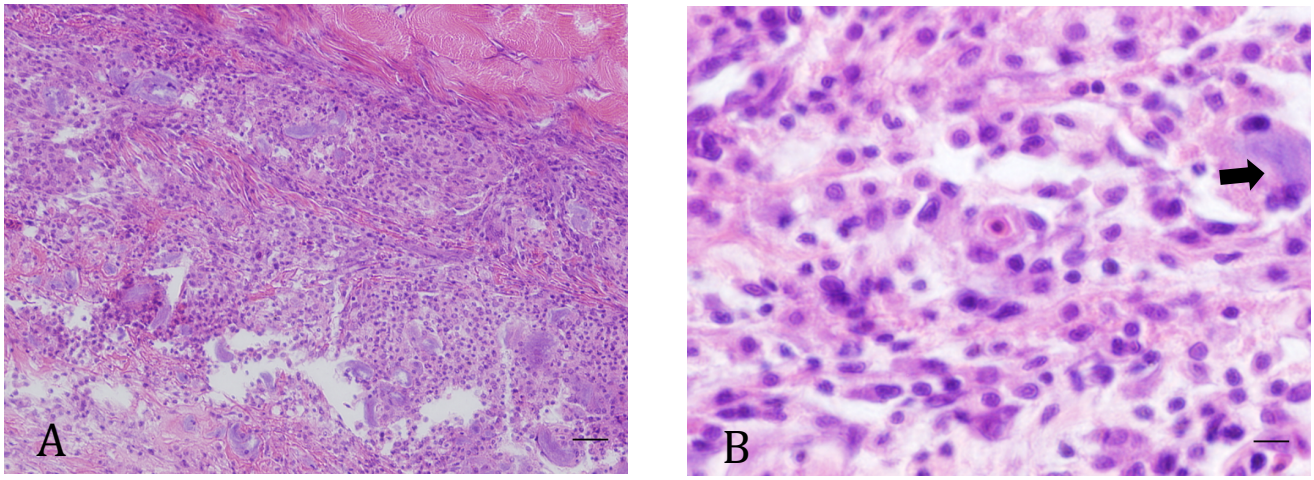

FIGURE 9. Inflammation in the injection sites of fish stimulated with heat-killed M30. A) A low magnification view of inflammation in an injection site. B) inflammation in an injection site with M30 particulate (arrow). Hematoxylin and eosin stain [bar = A) 50 µm, B) 5 µm]. Samples were collected one week (168 hr.) post-stimulation.

Histological Findings: Inflammation

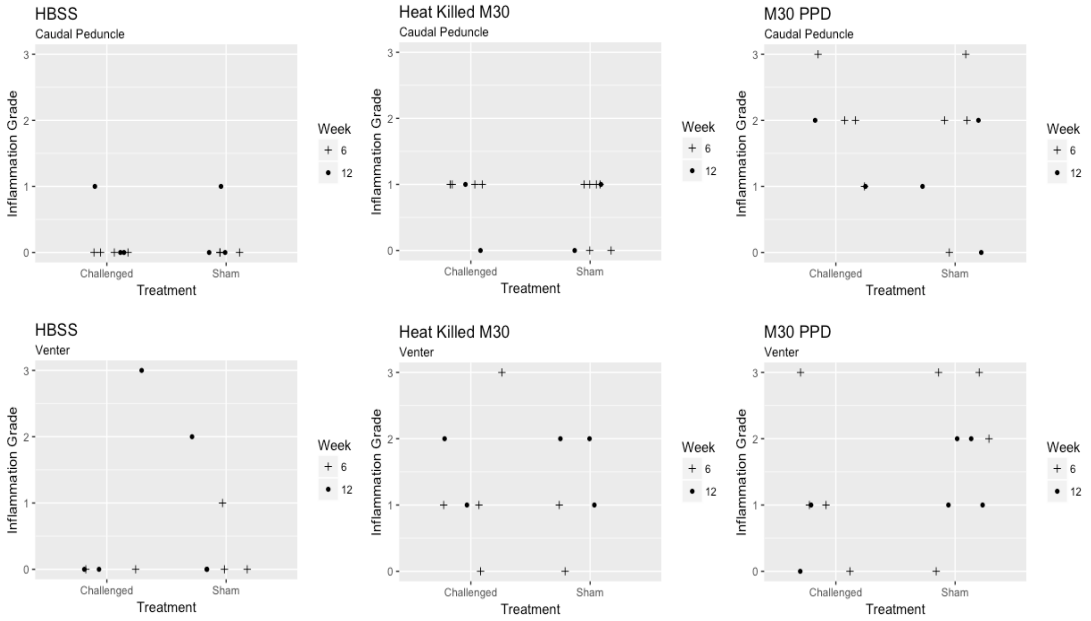

FIGURE 10. The grade of inflammation (0-3). Inflammation was graded in the caudal peduncle and venter of sham-exposed and challenged fish stimulated with HBSS, or heat-killed M30 and M30 PPD 6 and 12 weeks post-injection. Inflammation was graded as: (0) no presence of inflammation; inflammation comprising 0-10 % (1), 10-50 % (2), or 50-100 % (3) of abnormal tissue. Samples were collected one week (168 hr.) post-stimulation.

Myodegeneration

Most fish stimulated with M30 PPD or heat-killed M30 displayed mild to severe myodegeneration (shown in Figure 11) in the injection site, while this change was rarely observed in HBSS stimulated fish (Figure 12). There was no difference in the grade of myodegeneration in sham-exposed and challenged fish stimulated with HBSS, heat-killed M30, or M30 PPD 6 and 12 weeks post-infection [Kruskal-Wallis Rank Test (6wk & 12wk, caudal peduncle): All treatment comparisons, df = 1, $p > 0.05$; (6wk & 12wk, venter): All treatment comparisons, df = 1, $p > 0.05$].

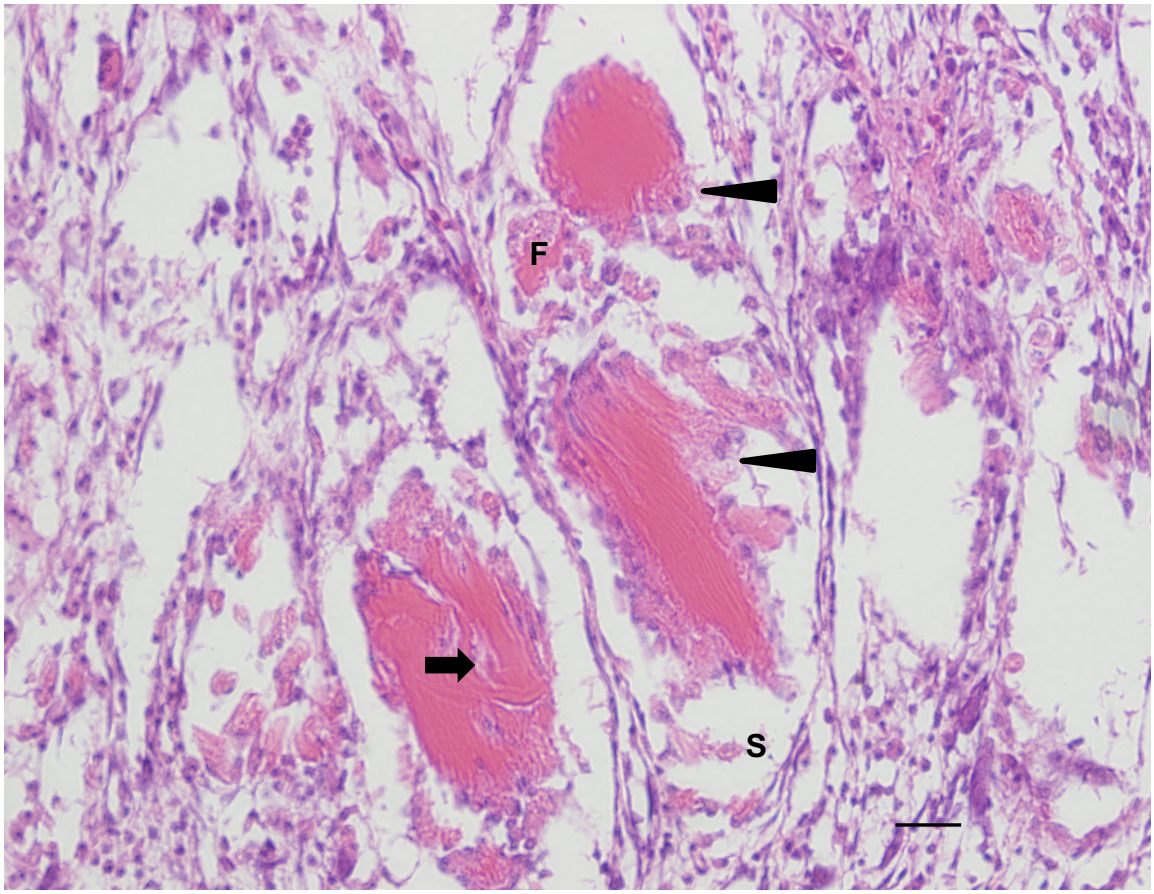

FIGURE 11. Myodegeneration seen in mycobacteria antigen-stimulated fish. The presence of myophagocytosis and cellular invasion of the muscle fibers are identified with arrowheads. Swollen (S), hyalinized (arrow) and fragmented muscle fibers (F) are also observed. Hematoxylin and eosin stain (bar = 50 µm). Samples were collected one week (168 hr.) post-stimulation.

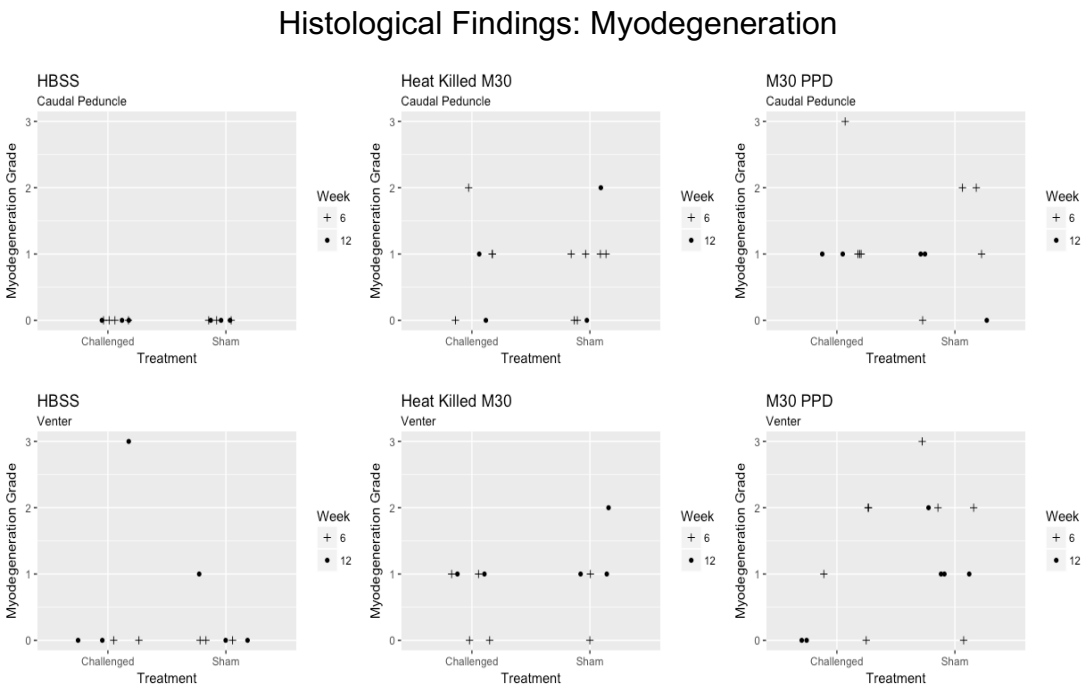

FIGURE 12. The grade of myodegeneration (0-3). Myodegeneration was graded in the caudal peduncle and venter of sham-exposed and challenged fish stimulated with HBSS or heat-killed M30 and M30 PPD 6 and 12 weeks post-injection. Myodegeneration was graded as: (0) absent; present comprising 0-10 % (1), 10-50 % (2), or 50-100 % (3) of abnormal tissue. Samples were collected one week (168 hr.) post-stimulation.

Myoregeneration

Myoregeneration was observed in most M30 PPD-stimulated fish, and few M30 heat-killed and HBSS stimulated fish (Figures 13 & 14). There was no difference in the grade of myoregeneration in sham-exposed and challenged fish stimulated with HBSS, heat-killed M30, or M30 PPD 6 and 12 weeks post-infection [Kruskal-Wallis Rank Test (6wk & 12wk, caudal peduncle): All treatment comparisons, df = 1, $p > 0.05$; (6wk & 12wk, venter): All treatment comparisons, df = 1, $p > 0.05$].

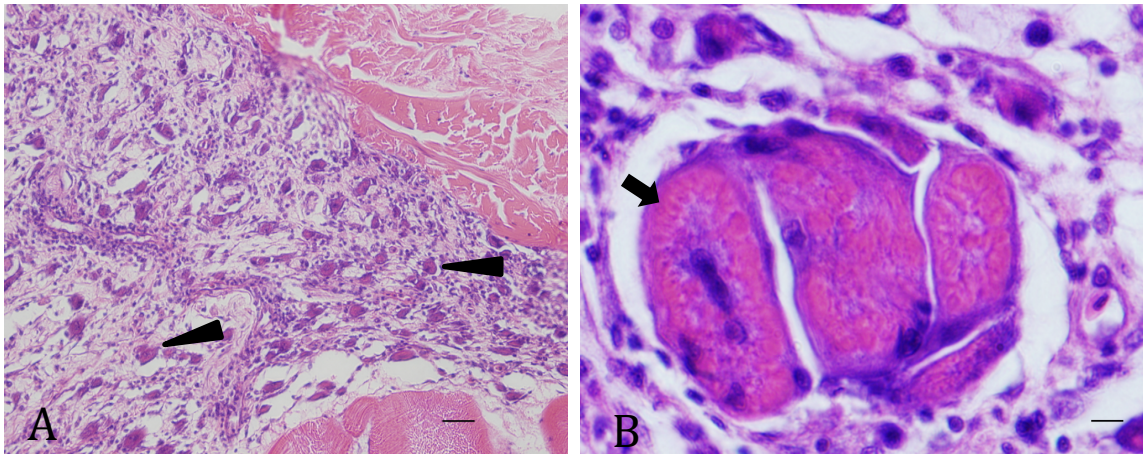

FIGURE 13. Myoregeneration seen in mycobacteria antigen-stimulated fish. A) A low magnification view of myoregeneration identified via the presences of myoblasts (arrowheads). B) A high magnification view of actomyosin bundles (arrow) in myoblasts. Hematoxylin and eosin stain [bar = A) 50 μm, B) 5 μm]. Samples were collected one week (168 hr.) post-stimulation.

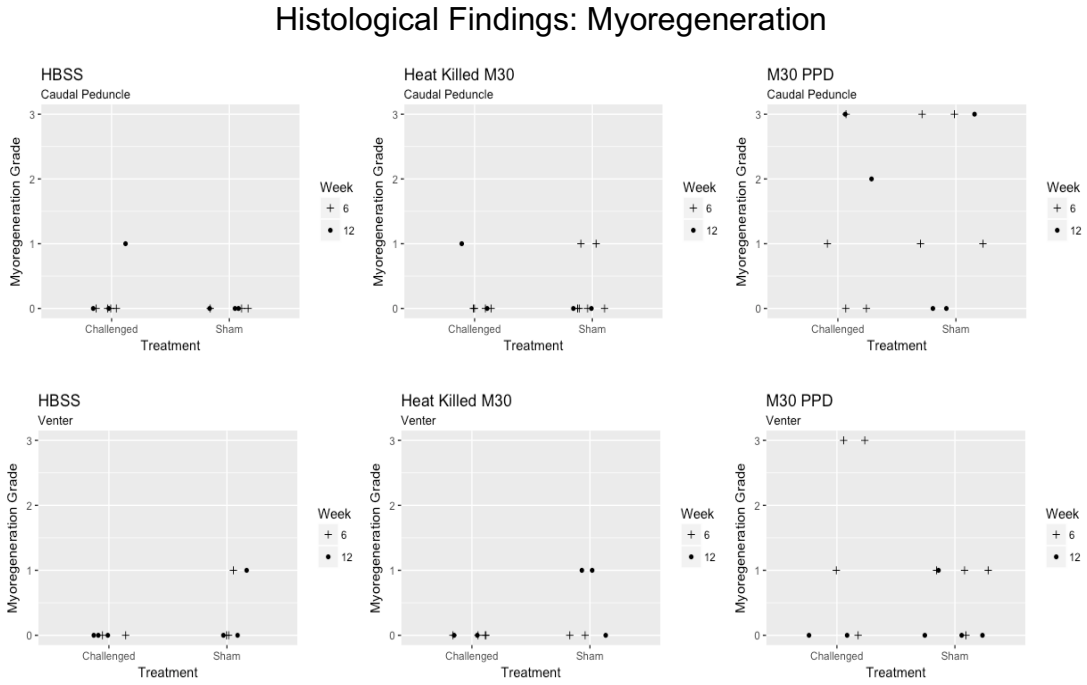

FIGURE 14. The grade of myoregeneration (0-3). Myoregeneration was graded in the caudal peduncle and venter of sham-exposed and challenged fish stimulated with HBSS or heat-killed M30 and M30 PPD 6 and 12 weeks post-injection. Myoregeneration was graded as: (0) absent; present comprising 0-10 % (1), 10-50 % (2), or 50-100 % (3) of abnormal tissue. Samples were collected one week (168 hr.) post-stimulation.

Hemorrhage and Granulomas

Qualitatively, the presence of hemorrhage (Figure 15) was seen more frequently in fish stimulated with heat-killed M30 and M30 PPD (Figure 16). The presence of granulomas was observed predominantly in fish that were challenged (Figure 17). There was no difference in the presence of hemorrhage or in the presence of granulomas in the caudal peduncle and venter sites of 6 and 12 week post-injected fish stimulated with HBSS or heat-killed M30 and M30 PPD among treatment groups (All treatment comparisons: Fisher's Exact Test; confidence level = 0.95, $p > 0.05$).

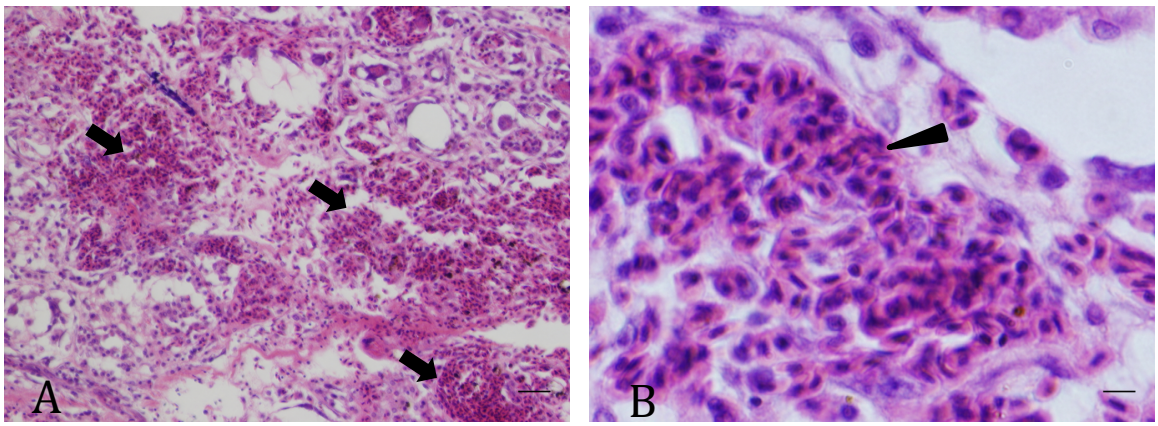

FIGURE 15. Hemorrhage seen in mycobacteria antigen-stimulated fish. A) A low magnification view of the presence of hemorrhage (arrow) in mycobacterial antigen stimulated fish. B) A high magnification view of an accumulation of erythrocytes (arrowhead). Hematoxylin and eosin stain [bar = A) 50 μm, B) 5 μm]. Samples were collected a week post-stimulation.

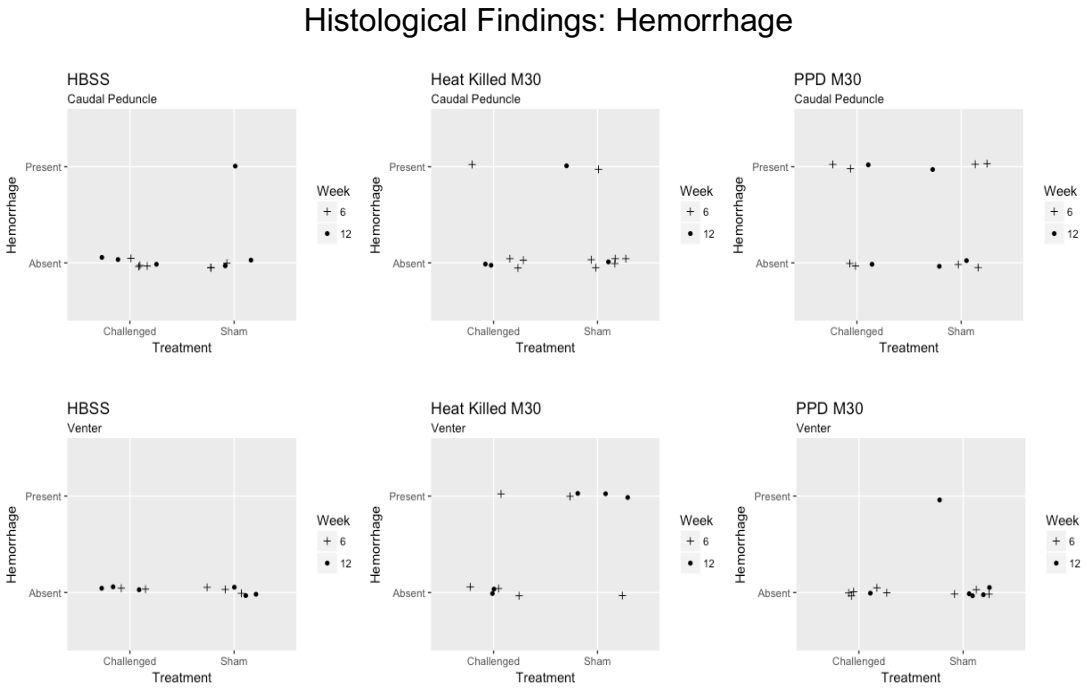

FIGURE 16. The presence of hemorrhage. Hemorrhage was seen in the caudal peduncle and venter of sham-exposed and challenged fish stimulated with HBSS or heat-killed M30 and M30 PPD 6 and 12 weeks post-injection. Samples were collected a 168 hr. post-stimulation.

FIGURE 17. The presence of granulomas. Granulomas were seen in the caudal peduncle and venter of sham-exposed and challenged fish stimulated with HBSS or heat-killed M30 and M30 PPD 6 and 12 weeks post-injection. Samples were collected 168 hr. post-stimulation.

STRIPED BASS IFN-γ GENE

The Striped Bass IFN-γ gene extracted and assembled from deposited transcriptomic data is estimated to be 6,245 base pairs (bp) long. The IFN-γ gene contains four exons (230 amino acids in total). Putative elements of the 5' and 3' UTR such as the TATA box, internal ribosome entry site (IRES), polyadenylation sites (PSA), and cytoplasmic polyadenylation elements (CPE) were identified (Figure 18) (Pesole et al. 2001; Furnes et al. 2009).

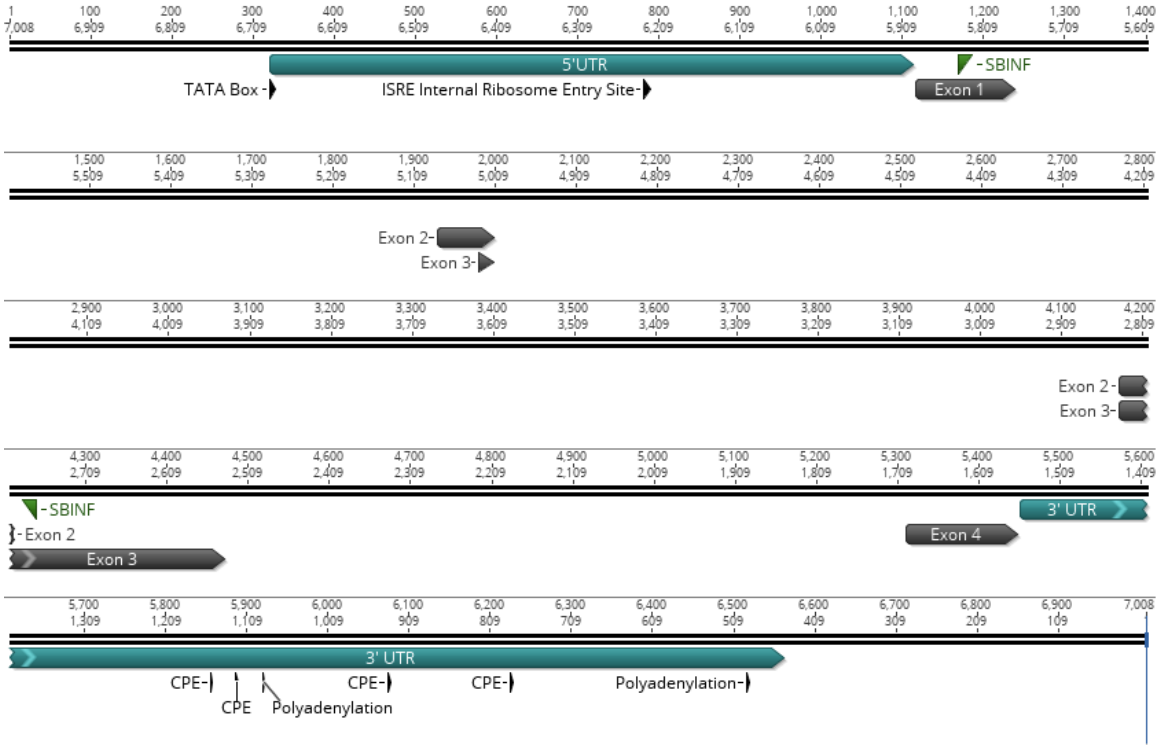

FIGURE 18. The Striped Bass IFN-γ gene. The gene is approximately 6,245 base pairs (bp) long and contains four exons. The putative elements of the 5' and 3' UTR such as the TATA box, internal ribosome entry site (IRES), polyadenylation sites (PSA), and cytoplasmic polyadenylation elements (CPE) are labeled. The Striped Bass IFN-γ primers (SBIFN) for RT-qPCR are labeled and span exons 1 through 3.

Nucleotide and amino acid pairwise identity of the Striped Bass IN-γ mRNA was determined by comparison to annotated IFN-γ mRNA of other fishes (Table 5). The Striped Bass IFN-γ mRNA is similar to European Seabass (*Dicentrarchus labrax*) with a 92.0 % nucleotide pairwise identity and a 88.4 % amino acid pairwise identity. The least identity of the Striped Bass IFN-γ mRNA was to Rainbow Trout (*Oncorhynchus mykiss*) having 54.3 % nucleotide pairwise identity and 29.3 % amino acid pairwise identity. The Striped Bass IFN-γ mRNA was also compared to Atlantic Halibut (*Hippoglossus hippoglossus*) (68.3 % nucleotide pairwise identity and 52.4 % amino acid pairwise identity). The

predicted secondary and tertiary protein structure of the Striped Bass IFN-γ mRNA was examined using Phyre2 (Figure 19). All protein comparisons had a 100 % confidence in the model predicted and a coverage of the query sequence that ranged from 60-66 % to the interferon/interleukin-10 (IL-10) family.

TABLE 5. IFN-γ nucleotide and amino acid pairwise identities. Pairwise identities to the Striped Bass IFN-γ mRNA were found using annotated IFN-γ genes from other fishes.

Species	Nucleotide Pairwise Identity	Amino Acid Pairwise Identity
European Seabass (*Dicentrarchus labrax*)	92.0 %	88.4 %
Halibut (*Hippoglossus hippoglossus*)	68.3 %	52.4 %
Rainbow Trout (*Oncorhynchus mykiss*)	54.3 %	29.3 %

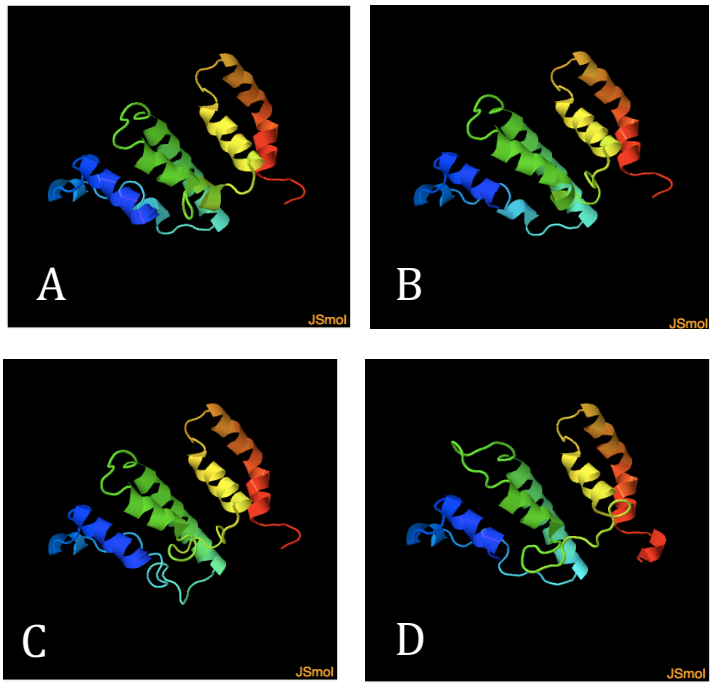

FIGURE 19. Predicted protein structures of IFN-γ mRNA of fishes. Models of secondary and tertiary protein structures were predicted using Phyre2. A) Striped Bass, B) European Sea Bass (*Dicentrarchus labrax*), C) Halibut (*Hippoglossus hippoglossus*), and D) Rainbow Trout (*Oncorhynchus mykiss*).

IN VITRO DTH ASSAY

The efficiencies of the housekeeping primers and SBIFN primers were all within optimal range (EF-1α) E = 107.8 %, R^2 = 0.937; (L9) E =102.7 %, R^2 = 0.989; and (SBIFN) E = 106.4 %, R^2 = 0.996. Neither PBL nor AK cells of any treatment group produced viable results via RT-qPCR. The Cq values were high (> 27) for all tested primers, and the melt curves were inconsistent for SBIFN primers suggesting the presence of primer dimerization (Cq Average and Median: L9, mean = 28.82, ±SD = 2.326751, median = 28.87; EF-1α, mean = 30.66, ±SD = 3.518968, median = 30.72; SBIFN, mean = 22.86, ±SD = 14.02957, median = 30.69). When testing PBL cells with SBIFN primers 69% of

the samples did not produce a Cq value. Control and PPD-H cells of AK tested with SBIFN primers did not produce a Cq value in 86 % of cases (Cq Average and Median: SBIFN, mean = 34.774, ±SD = 2.939608, median = 34.175). Further testing with housekeeping primers was deemed unnecessary due to high Cq values and inconsistent melt curves.

CONTAMINATION IDENTIFICATION

No mortalities occurred within two weeks of the initial inoculation with contaminated material in the primary study. Fish were therefore re-inoculated and the contaminants were identified. The best hits of the BLAST searches of the contaminants are as follows: *Acinetobacter* sp. (Genbank Accession #LT601029) coordinates 684-280 and 90.2 % pairwise identity; *Acinetobacter ursingii* (Genbank Accession #NR_025392) coordinates 657-273 and 90.5 % pairwise identity; uncultured *Xanthomonas* sp. (Genbank Accession #LT576256) coordinates 668-288 and 89.9 % pairwise identity; *Mycobacterium* sp. (Genbank Accession #KU172877) coordinates 642-259 and 95.6 % pairwise identity; *Mycobacterium intracellulare* (Genbank Accession #AY648870) coordinates 646-372 and 92.4% pairwise identity; *Rhodopseudomonas palustris* (Genbank Accession #EU808045) coordinates 459-424 and 100.0 % pairwise identity.

IV. DISCUSSION

NO EVIDENCE OF A DTH RESPONSE

No evidence of a Type IV hypersensitivity response was observed in the preliminary or primary *in vivo* studies of Striped Bass. During 3-7 days of monitoring and measuring the caudal peduncle of these fish after stimulation no indurations were observed and only minor hemorrhage at the injection site was seen. There was an increase in size in caudal width in six week sham-exposed fish post-stimulation with HBSS at 168 hours in the primary study; however, given that no such changes were seen in other treatment groups, this suggests measurement error or the possibility of an injection-site infection within this group of fish.

No evidence of DTH was seen histologically in the caudal peduncle and/or venter sites of fish stimulated with M30 PPD or heat-killed M30 6 and 12 weeks post-inoculation. There were no signs of induration or edema in the dermis, subcutaneous layer and/or muscle of any samples. There were other pathological changes such as inflammation, myodegeneration, myoregeneration, hemorrhaging, and granuloma formations around the stimulant injection site; however, these changes were similar in fish infected with M30 and sham-exposed with HBSS. Histologically, *M. marinum* particulate was observed in fish stimulated with heat-killed M30 but was not observed in fish stimulated with M30 PPD (soluble proteins). Myoregeneration was more common in the abnormal areas of fish stimulated with M30 PPD than fish stimulated with heat-killed M30. This suggests that M30 PPD can inflict tissue damage and that the fish stimulated with M30 PPD can remove the soluble antigen more effectively than the particulate material of heat-killed M30.

POTENTIAL REASONS FOR LACK OF DTH RESPONSE

There are few observations of DTH reactions recorded in fishes, and the presence of this reaction across teleosts is poorly defined. Due to increased

genome sequencing, the absence of major mammalian immune pathways has been observed with increasing frequency in fishes such as Gadidae, Agnathans, and Sygnathidae (Matsunaga and Rahman 1998; Pilström et al. 2005; Rombout et al. 2005; Cooper and Alder 2006; Star et al. 2011; Rauta et al. 2012; Star and Jentoft 2012; Haase et al. 2013). For example, the genes that code for major histocompatibility complex (MHC) and T-cell receptor (TCR) have not been observed in the primitive Agnathans (Pancer et al. 2004; Alder et al. 2005; Cooper and Alder 2006). However, agnathans are still capable of an adaptive immune response, as demonstrated by Finstad and Good (1964), as they possess variable lymphocyte receptors used to generate diverse antigen recognition pathways receptors (Pancer et al. 2004; Alder et al. 2005; Cooper and Alder 2006). Genomic research has revealed that Atlantic Cod lack the genes for MHC II, CD4, and invariant chain (Ii) which are all essential for T helper cell activation in higher vertebrates (Pilström et al. 2005; Star et al. 2011). Genes that encode CD4 and MHC II α and β chain are also absent in the Broadnosed Pipefish (*Syngnathus typhle*) (Haase et al. 2013). The Big Belly seahorse (*Hippocampus abdominalis*) only possesses a single functional MHC class IIβ-gene locus (Bahr and Wilson 2012; Haase et al. 2013).

Striped Bass may be missing crucial elements of the DTH pathway as well, such as MHC II, CD4, or an interferon gamma receptor (IFNGR) which is predicted to aid in TH2-TH1 phenotype switching (Schroder et al. 2004). Humans with inactive or partially working IFNGR have been closely associated with susceptibility to mycobacteria infections (Jouanguy et al. 1996; Newport et al. 1996; Jouanguy et al. 1997; Jouanguy et al. 1997; Pierre-Audigier et al. 1997; Dorman and Holland 1998; Jouanguy et al. 1999; Roesler et al. 1999; Döfflnger et al. 2000; Schroder et al. 2004). We may be observing something similar in Chesapeake Bay Striped Bass, which have a high prevalence of mycobacteriosis compared to other fishes, including the closely related White Perch (*Morone americana*). A lack of DTH response in Striped Bass relative to other fishes is a plausible possibility due to the possibility of missing genetic elements of the DTH pathway for their apparent high susceptibility to mycobacterial infection.

The preliminary and primary studies are similar to previous piscine DTH studies; however, *M. marinum* has not previously been used for infection or as a DTH stimulant. Previous studies in fishes that demonstrated DTH reactions used *M. tuberculosis* antigen to sensitize test subjects (Finstad and Good 1964; Papermaster et al. 1964; Bartos and Sommer 1981), and to elicit DTH responses. *Mycobacterium marinum* infected humans do develop a DTH response to *M. marinum* PPD; 76.19 % of *M. marinum*-infected people in one study produced an induration greater than 10 mm 48 hours after stimulation with *M. marinum* PPD (Jolly and Seabury 1972). Of other mycobacterial PPDs [i.e. *M. tuberculosis,* Group III-Battey (*M. intracellulare*), *M. kansasii*] tested in the same individuals, *M. marinum* PPD produced the largest induration in 33.3% of cases, indicating the antigens present in *M. marinum* PPD may be less effective at eliciting DTH responses than those from other species.

Human DTH responses may decrease over time in people that are immunocompromised or people with severe infections (Huebner et al. 1993). While fish in the preliminary study were tested for DTH 11 months post infection and fish in the primary study were tested for DTH 6 and 12 weeks post infections, both studies produced the same results. Therefore duration of infection did not appear to be a factor in the experimental outcomes. The possibility that Striped Bass may require longer than 24-48 hours stimulation to produce a response, as in mammals, was also examined in the primary study, where responses were recorded up to a week post stimulant injection. In the primary study, fish challenged with M30 had a severe granulomatous (grades 2-3) disease (Figure 8), and it is possible that the severity of disease would suppress DTH response. However fish in the preliminary study had a milder splenic disease (Figure 7) and also did not produce a DTH response, suggesting severe infections did not inhibit DTH. Although there was no gross or histological evidence of a DTH response in Striped Bass of the primary study, pathological changes were observed histologically. Inflammation, myodegeneration, myoregeneration, hemorrhage, and granulomas were observed in stimulation sites of heat-killed M30 and M30 PPD in sham-exposed and challenged fish. This indicates that Striped Bass in

this study were not immunocompromised generally but were but did not specifically produce a classical DTH response.

In most histological studies of DTH reactions in mammals, the stimulated area is normally excised within 24 to 48 hours post stimulation to an antigen (Poulter et al. 1982; Offner et al. 1985; Black 1999). Removing the injection sites from Striped Bass 168 hours post stimulation may have prevented the histological observation of edema. Within histological samples of both the caudal peduncle and venter sites of the primary study there was no trace of an increase of fibrous elements associated with an induration which occurs with a DTH response (Stevenson and Raymond 1990; Huebner et al. 1993).

PROBLEMS WITH *IN VIVO* STUDY

When the primary fish were initially injected with contaminated inoculum the fish were observed for death and or abnormalities for two weeks. During this time no fatalities and/ or abnormalities occurred. Contaminating bacteria were identified as *Acinetobacter, Xanthomonas, Rhodopseudomonas, Mycobacterium intracellulare,* and *Mycobacterium* spp. *Acinetobacter* is an extracellular pathogen that will produce clinical signs of infection in fish within a week post-exposure in experimental studies (Nemec et al. 2001; Munoz-Price and Weinstein 2008; Kozińska et al. 2014). Unlike mycobacteria *Acinetobacter* affects the skin, gills and digestive tract of the host fish (Čož-Rakovac et al. 2002; Kozińska et al. 2014). Also, *Acinetobacter ursingii* is a human pathogen and as of yet has not been recorded in fish (Nemec et al. 2001). The gram negative bacteria, *Xanthomonas*, are predominantly plant pathogens and are rarely found in other environments (Hayward 1993). *Rhodopseudomonas palustris* is nonpathogenic and ubiquitous in nature and is currently being used as diet for aquaculture due to its high protein content (Kim and Lee 2000; Larimer et al. 2004). The bacterium identified that raised concern was *Mycobacterium intracellulare* and *Mycobacterium* sp. because they have similar pathobiology as *M. marinum* and could possibly cross react with the *in vivo* and *in vitro* DTH assays. We observed no DTH response in control fish, therefore potential

presence of contaminating *Mycobacterium* does not invalidate our experimental results.

PROGRESS OF DEVELOPING A NON-LETHAL ASSAY

Creating an *in vitro* assay to detect the transcription of IFN-γ in Striped Bass depends on sequencing, annotating and well annotated IFN-γ genes of other fishes to publicly available Striped Bass transcriptomic data and the Striped Bass genome. From the newly annotated gene, SBIFN primers were created. Housekeeping primers (EF-1α and L9) were selected from published literature. Before beginning *in vitro* assay trials, efficiencies were established for all primers to ensure accurate relative quantitation of the mRNA targets among all three primer sets.

A preliminary run of the *in vitro* assay was performed with both anterior kidney (AK) and PBL isolated leukocytes from the primary study. The three selected primer sets (EF-1α, L9, and SBIFN) were tested with unexposed and mycobacterial antigen exposed isolated leukocytes and did not provide useful results. Ultimately the low yields of RNA obtained from AK and PBL isolated leucocytes hampered the ability to produce meaningful Cq values with these primers; making it difficult to distinguish if high Cq values of IFN-γ were due to the lack of RNA or to the low presence of the target gene transcription. Whether Striped Bass have a DTH response or not is still inconclusive when tested *in vitro*.

V. CONCLUSION

No evidence of a DTH response was observed either grossly or histologically in our studies performed on Striped Bass. Only minor hemorrhage at the stimulant injection sites were observed. The duration of infection before stimulation and the presence of severe infection in the primary study were determined not to be factors in the experimental outcomes because the preliminary and primary study produced similar results. Although a classical DTH response was not observed histologically, pathological changes such as inflammation, myodegeneration, myoregenereation, hemorrhage, and granulomas were present in all treatment groups, indicating that the Striped Bass were not immunocompromised. Ultimately the preliminary and primary *in vivo* DTH studies were not able to induce an *in vivo* DTH response in *M. marinum* infected striped bass stimulated with either heat-killed *M. marinum* or *M. marinum* PPD.

The preliminary trial of the *in vitro* assay, designed to detect the transcription of IFN-γ using RT-qPCR, performed in the primary study was unsuccessful. The lack of RNA obtained from isolated leukocytes stimulated *in vitro* resulted in unreliable Cq values. The selected primers (EF-1α, L9, and SBIFN) did prove to be efficient and repeatable when tested with a sufficient amount of Striped Bass RNA. Unfortunately it is still unknown if Striped Bass have a DTH response when tested *in vitro*.

FUTURE DIRECTIONS

The TST is widely used today to determine if an individual is infected or has been exposed to TB, but is impractical for uses other than general health care. The TST can be an inconvenience for research purposes due to having to observe a test subject more than once. There is an increased risk of cross reactivity with NTM infections and those vaccinated with BCG when performing a TST (Huebner et al. 1993; Diel et al. 2009; Slater et al. 2013; Manuel et al. 2007). The booster effect that occurs in frequent TST tests performed on

individuals who work in health care is also a concern with the TST, thus it is being replaced with IFN-γ release assays (Slater et al. 2013). An IFN-γ release assay such as the QuantiFERON-TB Gold assay is performed *in vitro* thus multiple observations of a subject are not necessary and the booster effect can be avoided. The use of specific proteins that are not present in BCG, as stimulants in the QuantiFERON-TB Gold assay decreases the probability of cross reactions with NTM infections and BCG vaccinated individuals (Farhat et al. 2006; Manuel et al. 2007; Connell et al. 2008; Diel et al. 2009; Slater et al. 2013; Pai et al. 2014).

In vitro IFN-γ release assays are also becoming popular in disease ecology of free-ranging mammals. The practicality and efficiency of an *in vitro* assay is reducing the need to perform recapture studies and/or holding tested animals, as needed when performing an *in vivo* DTH assay, to determine the effects of a disease on a population (Grobler et al. 2002). *In vitro* IFN-γ release assays have been used to detect *M. bovis* infections in European Badgers *(Meles* meles) and in African buffaloes (*Syncerus caffer*) (Grobler et al. 2002; Sawyer et al. 2007; Parsons 2010; Parsons et al. 2011). This technology could be used detect mycobacterial infections and or exposure in fishes.

Currently there are no non-lethal assays to detect mycobacteriosis fishes thus detection requires lethal sampling to obtain histological or bacteriological samples of the visceral organs. The development of an IFN-γ release assay for fishes would assist aquaculture, recreational, and commercial fisheries. This assay would allow aquaculturists and aquarists to remove infected individuals within a stock or tank preventing future outbreaks or the culling of an entire stock or highly valued aquarium specimens (Beran et al. 2006; Gauthier and Rhodes 2009). Field researchers could more effectively obtain data on disease status of fish populations via collecting blood samples rather than lethal sampling. Researchers would then have more insight into the prevalence of mycobacteriosis and its effects on wild populations of fishes without having to perform a long term recapture study. An IFN-γ release assay for fishes could

assist in disease ecology research in the prevalence of mycobacteriosis and improve biosecurity and broodstock screening.

RECOMMENDATIONS FOR FUTURE STUDIES

To further the development of a non-lethal diagnostic assay to detect mycobacteriosis in fishes testing different fishes for a DTH response is critical. A DTH response should be established *in vivo* in a fish before testing *in vitro* for assurance that the fish tested is capable of a DTH response. For instance Rainbow Trout would be a good model species for an *in vitro* DTH assay because it was established that these fish will produce a DTH response when sensitized and stimulated with a mycobacterial antigen (Bartos and Sommer 1981). The use of *M. tuberculosis* as a control sensitizing agent is also recommend for future *in vivo* and *in vitro* DTH assays (Finstad and Good 1964; Papermaster et al. 1964; Bartos and Sommer 1981). Using different antigens to stimulate infected fish should be considered as well because some may elicit a greater DTH response than the stimulation from the antigen used to create infection (Jolly and Seabury 1972).

Once a fish is identified to produce an *in vivo* DTH response that fish can be used for the development of an *in vitro* DTH assay. When performing the *in vitro* DTH assay the pathogen used and how it may affect possible samples for collection should be considered. For instance, when obtaining samples the anterior kidney is normally chosen in immunologically studies because it hosts an abundance of leukocytes that can be placed in culture. However, in Striped Bass with severe infections of *M. marinum* M30, the anterior kidney was heavily impacted by the abundance of granulomas; thus it was difficult to collect viable leukocytes for *in vitro* testing. The collection of blood would be a more reliable source for isolating viable leukocytes for an *in vitro* assay.

The amount of isolated leukocytes necessary to yield an acceptable amount of RNA for RT-qPCR should also be considered before performing an *in vitro* DTH assay. The lack of RNA collected from exposed leukocytes was a major hindrance in developing an *in vitro* DTH assay in this primary study. Even

though housekeeping genes were detected from cells plated at 1×10^6 per well, the average Cq value was high. Increasing the number of cells per well for exposure enough to obtain 100 ng of RNA will assist in obtaining lower Cq values in housekeeping genes. When efficiencies were being established for primer sets L9 and EF-1α, 100 ng of RNA template was used resulting Cq averages of 22.16 (L9) and 23.79 (EF-1α) when run with undiluted RNA. Increasing the amount of cells per well and lowering the Cq values of the housekeeping genes will in turn increase the probability of detecting the regulation of IFN-γ via transcription.

Other avenues of developing a non-lethal assay to detect mycobacteriosis in fishes is to explore the fish genetically. Other immune components (e.g. MHC II) should be identified before beginning *in vivo* and *in vitro* DTH testing. Only determining if the fish possesses the gene for IFN-γ is unreliable because IFN-γ is also used in innate immunity (Davies et al. 1982; Schroder et al. 2004). Finding and characterizing the other key genetic components in the DTH pathway, such as MHC II, Ii, and/or CD4, could assist in determining if a fish is cable of producing a classical mammalian DTH response. This can be achieved with free published transcriptomic data and genomes before beginning an *in vivo* and *in vitro* DTH assay testing.

REFERENCES

Alder, M. N., I. B. Rogozin, L. M. Iyer, G. V. Glazko, M. D. Cooper and Z. Pancer. 2005. Diversity and function of adaptive immune receptors in a jawless vertebrate. Science 310:1970-1973.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers and D. J. Lipman. 1990. Basic local alignment search tool. Journal of Molecular Biology 215:403-410.

ASMFC. 2016. Atlantic States Marine Fisheries Commission Available:http:// www.asmfc.org/species/atlantic-striped-bass.

Bahr, A. and A. B. Wilson. 2012. The evolution of MHC diversity: evidence of intralocus gene conversion and recombination in a single-locus system. Gene 497:52-57.

Bartos, J. M. and C. V. Sommer. 1981. *In vivo* cell mediated immune response to *M. tuberculosis* and *M. salmoniphilum* in rainbow trout (*Salmo gairdneri*). Developmental & Comparative Immunology 5:75-83.

Baya, A. 1998. Gram positive bacteria and skin lesions in Chesapeake Bay fish. Proceedings of a workshop on causes and effects of ulcerative lesions in fish, Easton, MD. Maryland Department of Natural Resources Oxford, MD.

Beran, V., L. Matlova, L. Dvorska, P. Svastova and I. Pavlik. 2006. Distribution of mycobacteria in clinically healthy ornamental fish and their aquarium environment. Journal of Fish Diseases 29:383-393.

Black, C. A. 1999. Delayed type hypersensitivity: current theories with a historic perspective. Dermatology Online Journal 5:7.

Cardinal, J. L. 2001. Mycobacteriosis in striped bass, *Morone saxatilis*, from Virginia waters of Chesapeake Bay. Master's Thesis, Virginia Institute of Marine Science.

Chinabut, S. 1999. Mycobacteriosis and nocardiosis. Pages 319-340 *in* P. T. K. Woo and D. W. Bruno, *editors*. CABI Publishing, New York, NY.

Connell, T. G., N. Ritz, G. A. Paxton, J. P. Buttery, N. Curtis and S. C. Ranganathan. 2008. A three-way comparison of tuberculin skin testing, QuantiFERON-TB Gold and T-SPOT.*TB* in children. PLoS One 3:e2624.

Cooper, M. D. and M. N. Alder. 2006. The evolution of adaptive immune systems. Cell 124:815-822.

Cotran, R. S., V. Kumar and T. Collins. 1999. Robbins pathologic basis of disease W.B. Saunders Company, Philadelphia, PA.

Čož-Rakovac, R., I. Strunjak-Perović, N. Topić Popović, M. Hacmanjek, B. Šimpraga and E. Teskeredžić. 2002. Health status of wild and cultured sea bass in the northern Adriatic Sea. Veterinární Medicína 47:222-226.

Crowle, A. J., R. Dahl, E. Ross and M. H. May. 1991. Evidence that vesicles containing living, virulent *Mycobacterium tuberculosis* or *Mycobacterium avium* in cultured human macrophages are not acidic. Infection and Immunity 59:1823-1831.

Davies, E., D. Isaacs and R. Levinsky. 1982. Defective immune interferon production and natural killer activity associated with poor neutrophil mobility and delayed umbilical cord separation. Clinical and Experimental Immunology 50:454-460.

Declercq, A. M., F. Haesebrouck, W. Broeck, P. Bossier and A. Decostere. 2013. Columnaris disease in fish: a review with emphasis on bacterium-host interactions. Veterinary Research 44:1-27.

Delves, P. J., S. J. Martin, D. R. Burton and I. M. Roitt. 2011. Roitt's Essential Immunology. Wiley-Blackwell, Hoboken, NJ.

Diel, R., R. Loddenkemper, K. Meywald-Walter, R. Gottschalk and A. Nienhaus. 2009. Comparative performance of tuberculin skin test, QuantiFERON-TB-Gold In Tube assay, and T-Spot.*TB* test in contact investigations for tuberculosis. Chest 135:1010-1018.

Döfflnger, R., E. Jouanguy, S. Dupuis, M. Fondanèche, J. Stephan, J. Emile, S. Lamhamedi-Cherradi, F. Altare, A. Pallier and G. Barcenas-Morales. 2000. Partial interferon-γ receptor signaling chain deficiency in a patient with bacille Calmette-Guérin and *Mycobacterium abscessus* infection. The Journal of Infectious Diseases 181:379-384.

Doig, K. D., K. E. Holt, J. A. Fyfe, C. J. Lavender, M. Eddyani, F. Portaels, D. Yeboah-Manu, G. Pluschke, T. Seemann and T. P. Stinear. 2012. On the origin of *Mycobacterium ulcerans*, the causative agent of Buruli ulcer. BMC Genomics 13:258.

Dorman, S. E. and S. M. Holland. 1998. Mutation in the signal-transducing chain of the interferon-gamma receptor and susceptibility to mycobacterial infection. The Journal of Clinical Investigation 101:2364-2369.

Drummond, A., B. Ashton, S. Buxton, M. Cheung, A. Cooper, C. Duran, M. Field, J. Heled, M. Kearse and S. Markowitz. 2010. Geneious v5.1. Biomatters Ltd. Available: www.geneious.com.

Dunn, O. J. 1964. Multiple comparisons using rank sums. Technometrics 6:241-252.

Dunnett, C. W. 1955. A multiple comparison procedure for comparing several treatments with a control. Journal of the American Statistical Association 50:1096-1121.

Farhat, M., C. Greenaway, M. Pai and D. Menzies. 2006. False-positive tuberculin skin tests: what is the absolute effect of BCG and non-tuberculous mycobacteria? The International Journal of Tuberculosis and Lung Disease 10:1192-1204.

Finstad, J. and R. A. Good. 1964. The evolution of the immune response: III. immunologic responses in the lamprey. The Journal of Experimental Medicine 120:1151-1167.

Frerichs, G. 1993. Mycobacteriosis: nocardiosis. Pages 219-233 *in* V. Inglis, et al., *editors*. Blackwell Scientific, London, UK.

Furnes, C., M. Seppola and B. Robertsen. 2009. Molecular characterisation and expression analysis of interferon gamma in Atlantic cod (*Gadus morhua*). Fish and Shellfish Immunology 26:285-292.

Gauthier, D., R. Latour, D. Heisey, C. Bonzek, J. Gartland, E. Burge and W. Vogelbein. 2008. Mycobacteriosis-associated mortality in wild striped bass (*Morone saxatilis*) from Chesapeake Bay, USA. Ecological Applications 18:1718-1727.

Gauthier, D. T., D. D. Cartwright, C. L. Densmore, V. S. Blazer and C. A. Ottinger. 2003. Measurement of in vitro leucocyte mitogenesis in fish: ELISA based detection of the thymidine analogue 5’-bromo-2′-deoxyuridine. Fish and Shellfish Immunology 14:279-288.

Gauthier, D. T., A. M. Helenthal, M. W. Rhodes, W. K. Vogelbein and H. I. Kator. 2011. Characterization of photochromogenic *Mycobacterium* spp. from Chesapeake Bay striped bass *Morone saxatilis*. Diseases of Aquatic Organisms 95:113-124.

Gauthier, D. T. and M. W. Rhodes. 2009. Mycobacteriosis in fishes: a review. Veterinary Journal 180:33-47.

Gauthier, D. T., W. Vogelbein and C. Ottinger. 2004. Ultrastructure of *Mycobacterium marinum* granuloma in striped bass *Morone saxatilis*. Diseases of Aquatic Organisms 62:121-132.

Geist, J., I. Werner, K. J. Eder and C. M. Leutenegger. 2007. Comparisons of tissue-specific transcription of stress response genes with whole animal endpoints of adverse effect in striped bass (*Morone saxatilis*) following treatment with copper and esfenvalerate. Aquatic Toxicology 85:28-39.

Gomes, M. S., S. Paul, A. L. Moreira, R. Appelberg, M. Rabinovitch and G. Kaplan. 1999. Survival of *Mycobacterium avium* and *Mycobacterium tuberculosis* in acidified vacuoles of murine macrophages. Infection and Immunity 67:3199-3206.

Grobler, D., A. L. Michel, L. De Klerk and R. Bengis. 2002. The gamma-interferon test: its usefulness in a bovine tuberculosis survey in African buffaloes (*Syncerus caffer*) in the Kruger National Park. Onderstepoort Journal of Veterinary Research 69:221-227.

Groner, M. L., Hoenig, J.M., Pradel, R., Choquet, R., Vogelbein, W.K., Gauthier, D.T., Friedrichs, M.A.M. 2018. Dermal mycobacteriosis and warming sea surface temperatures are associated with elevated mortality of striped bass in Chesapeake Bay. Ecology and Evolution 8:9384-9397.

Haase, D., O. Roth, M. Kalbe, G. Schmiedeskamp, J. P. Scharsack, P. Rosenstiel and T. B. Reusch. 2013. Absence of major histocompatibility complex class II mediated immunity in pipefish, *Syngnathus typhle*: evidence from deep transcriptome sequencing. Biology Letters 9:1-6.

Hayward, A. 1993. The hosts of *Xanthomonas*. Pages 1-119 *in* J. G. Swings and E. L. Civerolo, *editors*. Chapman and Hall, London, UK.

Hoenig, J. M., M. L. Groner, M. W. Smith, W. K. Vogelbein, D. M. Taylor, D. F. Landers Jr, J. T. Swenarton, D. T. Gauthier, P. Sadler and M. A. Matsche. 2017. Impact of disease on the survival of three commercially fished species. Ecological Applications 27:2116-2127.

Huebner, R. E., M. F. Schein and J. B. Bass Jr. 1993. The tuberculin skin test. Clinical Infectious Diseases 17:968-975.

Ivshina, M., P. Lasko and J. D. Richter. 2014. Cytoplasmic polyadenylation element binding proteins in development, health, and disease. Annual Review of Cell and Developmental Biology 30:393-415.

Jeon, S. J., L. C. Gonsalves, J. M. Jacobs, M. Rhodes, J. Councilman, A. Baya, E. B. May and M. D. Fast. 2011. Short-term infection of striped bass *Morone saxatilis* with *Mycobacterium marinum*. Diseases of Aquatic Organisms 94:117-124.

Jiang, H., K. H. Pollock, C. Brownie, J. M. Hoenig, R. J. Latour, B. K. Wells and J. E. Hightower. 2007. Tag return models allowing for harvest and catch and release: evidence of environmental and management impacts on striped bass fishing and natural mortality rates. North American Journal of Fisheries Management 27:387-396.

Jolly, H. W. and J. H. Seabury. 1972. Infections with *Mycobacterium marinum*. Archives of Dermatology 106:32-36.

Jouanguy, Emmanuelle, S. Lamhamedi-Cherradi, D. Lammas, S. E. Dorman, M. Fondanèche, S. Dupuis, R. Döffinger, F. Altare, J. Girdlestone and J. Emile. 1999. A human IFNGR1 small deletion hotspot associated with dominant susceptibility to mycobacterial infection. Nature Genetics 21:370-378.

Jouanguy, E., F. Altare, S. Lamhamedi, P. Revy, J. Emile, M. Newport, M. Levin, S. Blanche, E. Seboun and A. Fischer. 1996. Interferon-γ-receptor deficiency in an infant with fatal bacille Calmette-Guérin infection. New England Journal of Medicine 335:1956-1962.

Jouanguy, E., F. Altare, S. Lamhamedi-Cherradi and J. L. Casanova. 1997. Infections in IFNGR-1-deficient children. Journal of Interferon and Cytokine Research 17:583-587.

Jouanguy, E., S. Lamhamedi-Cherradi, F. Altare, M. Fondanèche, D. Tuerlinckx, S. Blanche, J. Emile, J. Gaillard, R. Schreiber and M. Levin. 1997. Partial interferon-gamma receptor 1 deficiency in a child with tuberculoid bacillus Calmette-Guerin infection and a sibling with clinical tuberculosis. The Journal of Clinical Investigation 100:2658-2664.

Kaattari, I., M. Rhodes, S. Kaattari and E. Shotts. 2006. The evolving story of *Mycobacterium tuberculosis* clade members detected in fish. Journal of Fish Diseases 29:509-520.

Kelley, L. A., S. Mezulis, C. M. Yates, M. N. Wass and M. J. Sternberg. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. Nature Protocols 10:845-858.

Kim, J. K. and B. Lee. 2000. Mass production of *Rhodopseudomonas palustris* as diet for aquaculture. Aquacultural Engineering 23:281-293.

Kozińska, A., E. Paździor, A. Pękala and W. Niemczuk. 2014. *Acinetobacter johnsonii* and *Acinetobacter lwoffii*-the emerging fish pathogens. Bulletin of the Veterinary Institute in Pulawy 58:193-199.

Kruskal, W. H. and W. A. Wallis. 1952. Use of ranks in one-criterion variance analysis. Journal of the American Statistical Association 47:583-621.

Larimer, F. W., P. Chain, L. Hauser, J. Lamerdin, S. Malfatti, L. Do, M. L. Land, D. A. Pelletier, J. T. Beatty and A. S. Lang. 2004. Complete genome sequence of the metabolically versatile photosynthetic bacterium *Rhodopseudomonas palustris*. Nature Biotechnology 22:55-61.

Li, C., B. H. Beck, S. A. Fuller and E. Peatman. 2014. Transcriptome annotation and marker discovery in white bass (*Morone chrysops*) and striped bass (*Morone saxatilis*). Animal Genetics 45:885-887.

Mahairas, G. G., P. J. Sabo, M. J. Hickey, D. C. Singh and C. K. Stover. 1996. Molecular analysis of genetic differences between *Mycobacterium bovis* BCG and virulent *M. bovis*. Journal of Bacteriology 178:1274-1282.

Manuel, O., A. Humar, J. Preiksaitis, K. Doucette, S. Shokoples, A. Peleg, I. Cobos and D. Kumar. 2007. Comparison of Quantiferon-TB-Gold with Tuberculin Skin Test for detecting latent tuberculosis infection prior to liver transplantation. American Journal of Transplantation 7:2797-2801.

Matsche, M. A., A. Overton, J. Jacobs, M. R. Rhodes and K. M. Rosemary. 2010. Low prevalence of splenic mycobacteriosis in migratory striped bass *Morone saxatilis* from North Carolina and Chesapeake Bay, U.S.A. Diseases of Aquatic Organisms 90:181-189.

Matsunaga, T. and A. Rahman. 1998. What brought the adaptive immune system to vertebrates? – the jaw hypothesis and the seahorse. Immunological Reviews 166:177-186.

Morrison, N. E. 1965. Circumvention of the mycobactin requirement of *Mycobacterium paratuberculosis*. Journal of Bacteriology 89:762-767.

Mosmann, T. R. and S. Sad. 1996. The expanding universe of T-cell subsets: Thl, Th2 and more. Immunology Today 17:138-146.

Munoz-Price, L. S. and R. A. Weinstein. 2008. *Acinetobacter* infection. New England Journal of Medicine 358:1271-1281.

Nemec, A., T. De Baere, I. Tjernberg, M. Vaneechoutte, T. Van Der Reijden and L. Dijkshoorn. 2001. *Acinetobacter ursingii* sp. nov. and *Acinetobacter schindleri* sp. nov., isolated from human clinical specimens. International Journal of Systematic and Evolutionary Microbiology 51:1891-1899.

Newport, M. J., C. M. Huxley, S. Huston, C. M. Hawrylowicz, B. A. Oostra, R. Williamson and M. Levin. 1996. A mutation in the interferon-γ–receptor gene and susceptibility to mycobacterial infection. New England Journal of Medicine 335:1941-1949.

NOAA. 2017. Striped Bass. NOAA Chesapeake Bay Office. Available:https://chesapeakebay.noaa.gov/fish-facts/striped-bass.

Offner, H., B. A. Standage, D. R. Burger and A. A. Vandenbark. 1985. Delayed type hypersensitivity to gangliosides in the Lewis rat. Journal of Neuroimmunology 9:147-157.

Ortiz, N. N., M. Gerdol, V. Stocchi, C. Marozzi, E. Randelli, C. Bernini, F. Buonocore, S. Picchietti, C. Papeschi and N. Sood. 2014. T cell transcripts and T

cell activities in the gills of the teleost fish sea bass (*Dicentrarchus labrax*). Developmental and Comparative Immunology 47:309-318.

Overton, A., F. Margraf, C. Weedon, L. Pieper and E. May. 2003. The prevalence of mycobacterial infections in striped bass in Chesapeake Bay. Fisheries Management and Ecology 10:301-308.

Pai, M., C. M. Denkinger, S. V. Kik, M. X. Rangaka, A. Zwerling, O. Oxlade, J. Z. Metcalfe, A. Cattamanchi, D. W. Dowdy and K. Dheda. 2014. Gamma interferon release assays for detection of *Mycobacterium tuberculosis* infection. Clinical Microbiology Reviews 27:3-20.

Pancer, Z., C. T. Amemiya, G. R. Ehrhardt, J. Ceitlin, G. L. Gartland and M. D. Cooper. 2004. Somatic diversification of variable lymphocyte receptors in the agnathan sea lamprey. Nature 430:174-180.

Papermaster, B. W., R. M. Condie, J. Finstad and R. A. Good. 1964. Evolution of the immune response. Journal of Experimental Medicine 119:105-130.

Papermaster, B. W., R. M. Condie, J. K. Finstad, R. A. Good and A. E. Gabrielsen. 1963. Phylogenetic development of adaptive immunity. Federation Proceedings 22:1152-1155.

Papermaster, B. W., R. M. Condie and R. A. Good. 1962. Immune response in the California hagfish. Nature 196:355-357.

Parsons, S. D., D. Cooper, A. J. McCall, W. A. McCall, E. M. Streicher, N. C. le Maitre, A. Müller, N. C. G. van Pittius, R. M. Warren and P. D. van Helden. 2011. Modification of the QuantiFERON-TB Gold (In-Tube) assay for the diagnosis of *Mycobacterium bovis* infection in African buffaloes (*Syncerus caffer*). Veterinary Immunology and Immunopathology 142:113-118.

Parsons, S. D. C. 2010. Natural animal model systems to study tuberculosis. Dissertation, Stellenbosch: University of Stellenbosch

Pauley, G. and C. Heartwell. 1983. Immune hypersensitivity in the channel catfish, *Ictalurus punctatus* (*Rafinesque*). Journal of Fish Biology 23:187-193.

Pesole, G., F. Mignone, C. Gissi, G. Grillo, F. Licciulli and S. Liuni. 2001. Structural and functional features of eukaryotic mRNA untranslated regions. Gene 276:73-81.

Pierre-Audigier, C., E. Jouanguy, S. Lamhamedi, F. Altare, J. Rauzier, V. Vincent, D. Canioni, J. Emile, A. Fischer and S. Blanche. 1997. Fatal disseminated *Mycobacterium smegmatis* infection in a child with inherited interferon γ receptor deficiency. Clinical Infectious Diseases 24:982-984.

Pilström, L., G. W. Warr and S. Strömberg. 2005. Why is the antibody response of Atlantic cod so poor? The search for a genetic explanation. Fisheries Science 71:961-971.

Poulter, L., G. Seymour, O. Duke, G. Janossy and G. Panayi. 1982. Immunohistological analysis of delayed-type hypersensitivity in man. Cellular Immunology 74:358-369.

Prophet, E. B., B. Mills, J. B. Arrington and L. H. Sobin. 1992. Laboratory methods in histotechnology. Armed Forces Institute of Pathology. American Registry of Pathology, Washington, D.C.

Rauta, P. R., B. Nayak and S. Das. 2012. Immune system and immune responses in fish and their role in comparative immunity study: a model for higher organisms. Immunology Letters 148:23-33.

Reading, B. J., D. A. Baltzegar, C. L. Dashiell, R. W. Chapman, J. Schilling, V. N. Williams, J. A. Macialek, B. D. Ring, M. Hopper, A. S. McGinty, R. J. Borski, S. McCoy, J. E. Schaff, C. H. Opperman and C. V. Sullivan. 2016. *Morone saxatilis* breed North Carolina State University Domesticated Broodstock SB-2011, whole genome shotgun sequencing project. North Carolina State University Available:https://www.ncbi.nlm.nih.gov/nuccore/JTCL00000000.1.

Rhodes, M. W., H. Kator, I. Kaattari, D. Gauthier, W. Vogelbein and C. A. Ottinger. 2004. Isolation and characterization of mycobacteria from striped bass *Morone saxatilis* from the Chesapeake Bay. Diseases of Aquatic Organisms 61:41-51.

Roesler, J., B. Kofink, J. Wendisch, S. Heyden, D. Paul, W. Friedrich, J. L. Casanova, W. Leupold, M. Gahr and A. Rösen-Wolff. 1999. *Listeria monocytogenes* and recurrent mycobacterial infections in a child with complete interferon-γ-receptor (IFNγR1) deficiency: mutational analysis and evaluation of therapeutic options. Experimental Hematology 27:1368-1374.

Rombout, J., H. Huttenhuis, S. Picchietti and G. Scapigliati. 2005. Phylogeny and ontogeny of fish leucocytes. Fish and Shellfish Immunology 19:441-455.

Savan, R., S. Ravichandran, J. R. Collins, M. Sakai and H. A. Young. 2009. Structural conservation of interferon gamma among vertebrates. Cytokine and Growth Factor Reviews 20:115-124.

Sawyer, J., D. Mealing, D. Dalley, D. Dave, S. Lesellier, S. Palmer, J. Bowen-Davies, T. Crawshaw and M. Chambers. 2007. Development and evaluation of a test for tuberculosis in live European badgers (*Meles meles*) based on measurement of gamma interferon mRNA by real-time PCR. Journal of Clinical Microbiology 45:2398-2403.

Schroder, K., P. J. Hertzog, T. Ravasi and D. A. Hume. 2004. Interferon-γ: an overview of signals, mechanisms and functions. Journal of Leukocyte Biology 75:163-189.

Slater, M. L., G. Welland, M. Pai, J. Parsonnet and N. Banaei. 2013. Challenges with QuantiFERON-TB Gold assay for large-scale, routine screening of US healthcare workers. American Journal of Respiratory and Critical Care Medicine 188:1005-1010.

Soman, S., B. V. Joseph, S. Sarojini, R. A. Kumar, V. Katoch and S. Mundayoor. 2007. Presence of region of difference 1 among clinical isolates of *Mycobacterium tuberculosis* from India. Journal of Clinical Microbiology 45:3480-3481.

Sørensen, A. L., S. Nagai, G. Houen, P. Andersen and A. B. Andersen. 1995. Purification and characterization of a low-molecular-mass T-cell antigen secreted by *Mycobacterium tuberculosis*. Infection and Immunity 63:1710-1717.

Star, B. and S. Jentoft. 2012. Why does the immune system of Atlantic cod lack MHC II? BioEssays 34:648-651.

Star, B., A. J. Nederbragt, S. Jentoft, U. Grimholt, M. Malmstrøm, T. F. Gregers, T. B. Rounge, J. Paulsen, M. H. Solbakken and A. Sharma. 2011. The genome sequence of Atlantic cod reveals a unique immune system. Nature 477:207-210.

Stevenson, R. M. W. and B. Raymond. 1990. Delayed-type hypersensitivity skin reactions. Pages 173-178 *in* J. S. Stolen, et al., *editors*. SOS Publications, Fair Haven, NJ.

Stinear, T. P., T. Seemann, S. Pidot, W. Frigui, G. Reysset, T. Garnier, G. Meurice, D. Simon, C. Bouchier and L. Ma. 2007. Reductive evolution and niche

adaptation inferred from the genome of *Mycobacterium ulcerans,* the causative agent of Buruli ulcer. Genome Research 17:192-200.

Teska, J. D., L. E. Twerdok, J. Beaman, M. Curry and R. A. Finch. 1997. Isolation of *Mycobacterium abscessus* from Japanese medaka. Journal of Aquatic Animal Health 9:234-238.

Upton, G. J. 1992. Fisher's exact test. Journal of the Royal Statistical Society. Series A (Statistics in Society) 155:395-402.

Vogelbein, W., D. Zwerner, H. Kator, M. Rhodes and J. Cardinal. 1999. Mycobacteriosis of striped bass from Chesapeake Bay. VIMS Special Scientific Report, 139, Gloucester Point, VA

Vogelbein, W., D. Zwerner, H. Kator, M. Rhodes, S. Kotob and M. Faisal. 1998. Mycobacteriosis in the striped bass, *Morone saxatilis*, from Chesapeake Bay. 3rd Symposium on Aquatic Animal Health, Baltimore, MD.

Wynne, J. W., B. J. Shiell, M. L. Colgrave, J. A. Vaughan, G. Beddome and W. P. Michalski. 2012. Production and proteomic characterisation of purified protein derivative from *Mycobacterium avium* subsp. *paratuberculosis*. Proteome Science 10:1-12.

VITA

Jessica Shannon Miller

EDUCATION
MS in Biological Sciences (Expected December 2018)
Old Dominion University, Norfolk VA 23529
Advisor: Dr. David Gauthier
Thesis title: *Lack of Delayed-Type Hypersensitivity in Striped Bass (Morone saxatilis)*
BS in Biology (May 2014)
Old Dominion University, Norfolk VA 23529

PROFESSIONAL POSITIONS
Graduate Teaching Assistant at Old Dominion University, Norfolk, VA (2015-2018)
Labs of Dr. Douglas Mills (Biological Sciences Dept.- General Biology Lab I and II), Dr. Jennifer Katz-Keenan (Biological Sciences Dept.- Introduction to Human Biology Lab, Fundamentals of Anatomy and Physiology Lab, and Human Cadaver Dissection)

AWARDS

- Graduate Student Travel Award; May 2018
- ODU Fellowship of Women in Science conference funding; May 2018
- Biology Graduate Student Organization conference funding; May 2017
- Third place for best presentation at the 2017 BGSO Symposium; April 2017
- Graduate Teaching Assistantship; August 2015-May 2018
- Certificate of Achievement: Graduate Teaching Assistant; August 2015
  Best Poster Presentation at American Fisheries Society: Fish and Health Section; July 2015

ORAL PRESENTATIONS

- **Miller, J. S.**, & Gauthier, D. T. (2018) Delayed-Type Hypersensitivity in Striped Bass (*Morone saxatilis*). Western Fish Disease Workshop, June. Bozeman, MT (Oral Presentation)
- **Miller, J. S.**, & Gauthier, D. T. (2017) Delayed-Type Hypersensitivity and Interferon-Gamma Response in Striped Bass (*Morone saxatilis*). Eastern Fish Health Workshop, April. East Lansing, MI (Oral Presentation)
- **Miller, J. S.**, & Gauthier, D. T. (2016) First Steps Toward an Interferon Capture Assay for Mycobacteriosis in Striped Bass (*Morone saxatilis*). Eastern Fish Health Workshop, April. Atlantic Beach, NC (Oral Presentation)