Tissue Localization of Piscidin Host-Defense Peptides during Striped Bass (*Morone saxatilis*) Development

Scott A. Salger[1*], Benjamin J. Reading[1], Edward J. Noga[2,3]

[1] Department of Applied Ecology, North Carolina State University, Raleigh, North Carolina, United States of America

[2] Department of Clinical Sciences, North Carolina State University, College of Veterinary Medicine, Raleigh, North Carolina, United States of America

[3] Current address: South Eastern Aquatechnologies, Inc., Boca Raton, Florida, United States of America

* Corresponding author

E-mail: sasalger@ncsu.edu (SAS)

Short Title: Tissue localization of fish host-defense peptides during development

Keywords: piscidin, striped bass, innate immunity, host-defense peptides, ontogeny

ABSTRACT

Infectious diseases are a major cause of larval mortality in finfish aquaculture. Understanding ontogeny of the fish immune system and thus developmental timing of protective immune tissues and cells, may help to decrease serious losses of larval fishes when they are particularly vulnerable to infection. One component of the innate immune system of fishes is the host-defense peptides, which include the piscidins. Piscidins are small, amphipathic, α-helical peptides with a broad-spectrum of action against viral, bacterial, fungal, and protozoan pathogens. We describe for the first time the cellular and tissue localization of three different piscidins (1, 3, and 4) during striped bass (*Morone saxatilis*) larval ontogeny using immunofluorescent histochemistry. From 16 days post hatch to 12 months of age, piscidin staining was observed in cells of the epithelial tissues of gill, digestive tract, and skin, mainly in mast cells. Staining was also seen in presumptive hematopoietic cells in the head kidney. The three piscidins showed variable cellular and tissue staining patterns, possibly relating to differences in tissue susceptibility or pathogen specificity. This furthers our observation that the piscidins are not a monolithic family of antimicrobials, but that different AMPs have different (more specialized) functions. Furthermore, no immunofluorescent staining of piscidins was observed in post-vitellogenic oocytes, embryos, or larvae from hatch to 14 days post hatch, indicating that this critical component of the innate immune system is inactive in pre-hatch and young larval striped bass.

1. INTRODUCTION

Diseases that lead to larval mortality significantly contribute to profit loss in the aquaculture industry and may influence year class recruitment in wild fisheries. The ability to fight bacterial infections before the adaptive immune system is developed is important to the survival of young fish. Embryos and larvae of fishes are exposed to many pathogens prior to the development and maturation of lymphoid organs and immunocompetence (Zapata et al. 2006). Maternal transfer of immune factors is a mechanism used by many organisms to protect the oocyte and/or egg and larva from infection. Several studies have shown the transfer of maternal immunoglobulins (Ig) to fish progeny through the yolk that is deposited into the oocytes during vitellogenesis (reviewed in Mulero et al. 2007b). These Igs may provide some level of protection against pathogens to the developing embryo and confer a higher level of survival to the offspring (Picchietti et al. 2006, Seppola et al. 2009, Hanif et al. 2004, Hayman et al 1993).

Innate immune factors also are present in the developing oocytes and embryos of fishes. Unfertilized and developing rainbow trout (*Oncorhynchus mykiss*) and carp (*Cyprinus carpio*) eggs were found to contain gene transcripts encoding proteins of the complement system, a component of the innate immune system that later plays a pivotal role in adaptive immunity (Løvoll et al. 2006, Huttenhuis et al 2006). Transcripts for lysozyme, cathelicidin (Seppola et al. 2009), and cod piscidins (Ruangsri et al. 2012), antimicrobial components of the innate immune system, appear to be transferred into the eggs of Atlantic cod (*Gadus morhua*). These factors may help to protect the developing embryos from vertically transmitted maternal bacterial pathogens. In the Atlantic cod studies, abundance of these gene transcripts decreased through time in the developing embryos and then later increased in the larvae. This may indicate that the

66 gene transcripts are transferred from the mother to the egg and then stored until the offspring can
67 begin to transcribe their own immune factors.
68 The ontogeny of the hematopoietic tissues and immune system in fish varies between
69 species. In the European sea bass (*Dicentrarchus labrax*), the head kidney is microscopically
70 visible 10 days post hatch (dph), the spleen at 18 dph, and the thymus at 21 dph (Abelli et al.
71 1996, Quesada et al. 1994), whereas in the Atlantic cod, they appear at 5 dph and 28 dph,
72 respectively (Schroder et al. 1998). Acidophilic granulocytes, the main phagocytic cell type in
73 vertebrates, were observed at 21 dph in the posterior intestine and blood, 27 dph in the kidney,
74 but not until 62 dph in the spleen and thymus in the gilthead seabream (*Sparus aurata*) (Mulero
75 et al. 2007a). Pre-T-cells were first seen 5-12 dph while pre-B-cells were not seen until 52 dph in
76 European sea bass, but levels of these immune cells were lower than typically observed in adult
77 fish and thus considered immunologically immature until 137-145 dph (dos Santos et al. 2000).
78 Also, IgM bearing cells were not detected until 38 dph in the head kidney of the same species
79 (Scapigliati et al 2002).
80 The ontogeny of the immune system of the striped bass (*Morone saxatilis*) has not been
81 well studied. Striped bass are an important aquacultured finfish species in the United States and
82 bred along with the white bass (*M. chrysops*) to create the hybrid striped bass (Garber and
83 Sullivan 2006). One component of the innate immune system is the host-defense peptides, also
84 known as antimicrobial peptides (AMPs). Host-defense peptides have a broad spectrum of
85 activity against microorganisms such as bacteria, fungi, parasites, and viruses (Diamond et al.
86 2009; Pálffy et al. 2009; Bulet et al. 2004). They have been identified in virtually all groups of
87 organisms, from bacteria to eukaryotic plants and animals. Their ubiquity and potent activity
88 suggest that they are critical to immune health. We have identified a family of host-defense

peptides in the striped bass named the piscidins (Colorni et al. 2008, Corrales et al. 2010, Salger et al. 2011, Salger et al. 2016, Silphaduang and Noga 2001). The piscidins are amphipathic, α-helical peptides between 22 and 55 amino acids in length and have been localized to mast cells in the gills, skin, and gut and in mast cells lining the blood vessels in the viscera of the hybrid striped bass (Corrales et al. 2010, Silphaduang and Noga 2001). They have been found to have potent antibacterial and antiprotozoan activities.

Knowing when and in which cells and tissues the piscidins first appear during striped bass development could help to decrease mortality due to disease in the culture of this species. This would also provide valuable knowledge of key protective tissue and cell development at important stages in the culture of the striped bass by identifying at which developmental stages the fish may be most vulnerable to infection and when the fish may develop the ability to defend itself from infection. Here we provide immunohistochemical analysis of the ontogeny of three piscidins (1, 3, and 4) during striped bass embryonic and larval development. We show that piscidins are not detected in post-vitellogenic oocytes or embryos and that they first appear in larvae between 14 and 16 dph. These three piscidins are not always produced and stored in the same cell types and tissues during the different stages of development in the fish. This furthers our observation that the piscidins are not a monolithic family of antimicrobials, but that various isoforms may have different (more specialized) functions.

2. MATERIALS AND METHODS

2.1 Experimental Animals

North Carolina State University Institutional Animal Care and Use Committee (IACUC) approved this research (IACUC #08-118-0). We euthanized all animals using MS-222 following standard procedures.

Striped bass (*Morone saxatilis*) were spawned as part of the *National Program for Genetic Improvement and Selective Breeding for the Hybrid Striped Bass Industry* at the North Carolina State University Pamlico Aquaculture Field Laboratory (PAFL) in Aurora, NC according to standard procedures. Post-vitellogenic oocyte biopsies were collected from mature female striped bass as described in Chapman et al. (2014). Embryos were collected from MacDonald hatching jars prior to hatching (approximately 36 hours post-fertilization). Hatchery phase fish [larvae at 1 hr post-hatch and 1 day post-hatch (dph), 3 dph, 5 dph, 7 dph, 9 dph] were collected from aquaria following hatching and swim up. Phase I fry (12 dph, 14 dph, 16 dph, 19 dph, 21 dph, and 24 dph) were collected from earthen ponds using either a plankton tow or light trap (MT3, Aquatic Ecosystems, Apopka, FL). Phase I fingerlings were collected from flow through tanks after they were harvested from earthen ponds at 27 dph, 33 dph, 41 dph, 45 dph, 49 dph, 55 dph, and 60 dph. Phase I fingerlings were transferred to North Carolina State University College of Veterinary Medicine (Raleigh, NC). The fish were held in 113.5 L aquaria fitted with a Triton sand filter (Pentair Aquatic Systems, Sanford, NC) and custom bioreactor. They were fed a commercial diet (Finfish Starter Meal to 5 mm Finfish Gold pellets, Zeigler Bros., Inc., Gardners, PA, depending on size of fish) and grown to Phase II size. Phase II fish were collected at 66 dph, 76 dph, 92 dph, 120 dph, 150 dph, and Phase III fish were collected at 12 months. Water temperature was monitored daily with ammonia, nitrite, pH, and salinity levels checked biweekly. Water exchanges were performed once daily.

2.2 Sample collection

Embryos were immediately placed into square mesh biopsy cassettes (#70073-W, Electron Microscopy Sciences, Hatfield, PA) and fry and fingerlings were similarly placed in Micromesh biopsy cassettes (#70074-W, Electron Microscopy Sciences). All fish were euthanized in buffered 200 mg/L MS-222 (Finquel, Argent Chemical Laboratories, Inc. Redmond, WA) and 400 mg/L sodium bicarbonate according to institutionally approved protocols. Twelve month old fish were euthanized in buffered 200 mg/L MS-222 and 400 mg/L sodium bicarbonate and the gill, stomach, anterior intestine, and skin was dissected and placed into a separate slotted histological cassette (#70071-W, Electron Microscopy Sciences). Samples for semi-quantitative reverse-transcription polymerase chain reaction (rt-PCR) studies were stored in RNAlater (Ambion, Austin, TX) until processing for gene expression. All samples for immunofluorescent histology were fixed in 10% neutral buffered formalin (Fisher Scientific, Inc., Waltham, MA) for 24 hr and rinsed with distilled water and stored in 70% ethanol until processing.

2.3 Primary Antibodies

Anti-piscidin antibodies were produced by a commercial laboratory (Bethyl Laboratories, Montgomery, TX) using the company's standard procedures (Corrales et al. 2010, Silphaduang et al. 2006). The following primary antibodies were used: 1) purified custom polyclonal antibodies against the amino terminal of striped bass piscidin 1 (rabbit anti-FFHH; peptide sequence – CFFHHIFRGIVH) at 0.6 mg/mL, 2) purified custom polyclonal antibodies against the amino terminal of striped bass piscidin 3 (rabbit anti-HAGR; peptide sequence – CHAGRSIGRFLTG) at 3.9 mg/mL, and 3) purified custom polyclonal antibodies against full

length striped bass piscidin 4 (rabbit anti-5.3; peptide sequence – FFRHLFRGAKAIFRGARQGWRAHKVVSRYRNRDVPETDNNQEEP) at 1.16 mg/mL.

2.4 Immunofluorescent histology

Fixed samples were processed for immunofluorescence histology following the methods of Dungan and Roberson (1993). Samples were embedded in paraffin, sectioned to 5 μm thickness with a microtome and mounted on Superfrost Plus slides (Fisher Scientific, Inc., Pittsburgh, PA) at the Histology Laboratory at the North Carolina State University College of Veterinary Medicine. Serial sections were either stained with hematoxalin and eosin (H&E) or left unstained for further immunofluorescent procedures.

Immunofluorescent detection of piscidins in striped bass histological sections was performed according to the procedures of Dungan and Roberson (1993), with some modifications. Slides were deparaffinized in xylene and rehydrated in graded ethanol and deionized water. After washing for 3 min in phosphate buffered saline (PBS; BP399-1, Fisher Scientific), sections were rinsed in blocking buffer [PBS with 0.05% Tween-20 (PBST) with 2.0% bovine serum albumin, 0.02% sodium azide and 0.2% normal goat serum] for 1 h at room temperature followed by washing for 2 min in PBST. Slides were then incubated in a humid chamber at room temperature with the appropriate primary antibody [rabbit anti-FFHH, 1:5 in serum diluent (PBST with 1.0% bovine serum albumin and 0.02% sodium azide); rabbit anti-HAGR, 1:50 in serum diluent; or rabbit anti-5.3, 1:1000 in serum diluent]. After 1 h, the slides were washed 3x in PBST for 2 min each. They were then incubated again for 1 h in a humid chamber at room temperature with the fluorophore-labeled secondary antibody (Alexa Fluor 488 goat anti-rabbit, Molecular Probes, Carlsbad, CA; 1:250 in serum diluent), followed by washing

180 twice in PBST and once in PBS for 2 min per wash. The sections were counterstained with
181 Hoechst 33342 (Molecular Probes; 1:100 in cold PBS) for 1 h in a humid chamber on ice. Each
182 slide was dipped 10 times in cold PBS and cover slipped using Prolong Gold (Molecular Probes,
183 Carlsbad, CA) anti-fade and mounting medium.

184 In all experiments, negative controls were performed by omitting either the primary
185 antibody, secondary antibody, both primary and secondary antibodies, or by substituting the
186 primary antibody with pre-immune serum from rabbits immunized with striped bass piscidin 1,
187 3, or 4 peptides.

188 Sections were examined using a Zeiss LSM 710 confocal unit on a Zeiss Axio Observer
189 Z1 microscope and Zen 2009 imaging software (Carl Zeiss, Inc., Thornwood, NY).
190 Immunofluorescent images were overlaid with their corresponding differential interference
191 contrast image at the North Carolina State University Department of Plant Biology Cellular and
192 Molecular Imaging Facility (Raleigh, NC).

193

194 *2.5 Semi-quantitative reverse-transcription polymerase chain reaction (rt-PCR)*

195 Piscidin mRNA gene expression was quantified using semi-quantitative rt-PCR
196 performed as previously described with a few exceptions (Reading et al. 2014; Williams et al.
197 2014). Briefly, total RNA was isolated from Post-vitellogenic oocytes, 36 hours post-fertilization
198 striped bass embryos, striped bass larvae at 1 hr post-hatch and 1 day post-hatch (dph), striped
199 bass fry at 3 dph, 5 dph, 7 dph, 9 dph, 12 dph, 14 dph, 16 dph, 19 dph, 21 dph, and 24 dph,
200 striped bass fingerlings at 27 dph, 33 dph, 41 dph, 45 dph, 49 dph, 55 dph, and 60 dph, striped
201 bass at 66 dph, 76 dph, 92 dph, 120 dph, and 150 dph, and gill, stomach, anterior intestine, and
202 skin from 12 months post hatch striped bass using Trizol reagent (Invitrogen, Carlsbad, CA).

One microgram of the total RNA was used to synthesize cDNA using a High Capacity cDNA Synthesis kit (Applied Biosystems, Carlsbad, CA) following treatment with Turbo DNA-free (Ambion, Foster City, CA). RNA was quantified and checked for quality at each step using a Nanodrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis, respectively. Gene expression of the different piscidin forms was measured in the striped bass cDNA using piscidin gene-specific primers (Invitrogen; striped bass piscidin 1: QP1F and QP1R primers; striped bass piscidin 3: QP3F and QP3R primers; striped bass piscidin 4: QP4F and QP4R1 primers; Salger et al. 2016). Specificity of the primers for each piscidin were verified by both forward and reverse direct sequencing of PCR products using hybrid striped bass gill and intestine cDNA as template. The control gene for the rt-PCR was *rpl9*.

3. RESULTS

No immunofluorescent staining with any of the antibodies used in this study (striped bass piscidins 1, 3, and 4) was observed in striped bass post-vitellogenic oocytes, embryos, or larvae at 1 hr post-hatch, 1 dph, 3 dph, 5 dph, 7 dph, 9 dph, 12 dph, or 14 dph (*data not shown*). The first appearance of immunofluorescent staining occurred at 16 dph or in phase I fish for all of the antibodies that were used. An overview hematoxylin and eosin (H&E) image of a 16 dph larval striped bass is shown for reference of tissues stated here (Fig. 1).

Staining using the antibody against striped bass piscidin 1 in the 16 dph larval sample was localized to mast cell progenitor cells in the buccal cavity (Fig. 2a) and hematopoietic cells in hematopoietic tissue of the head kidney (Fig. 2b) and mast cell progenitor cells in the dorsal aorta (Supplementary Fig. 1a). Striped bass piscidin 3 was localized to mast cell progenitor cells

226 in the kidney tubule epithelium (Supplementary Fig. 1b) and digestive epithelium
227 (Supplementary Fig. 1c) and mast cell progenitor cells in the dorsal aorta (Fig. 2c), and
228 hematopoietic cells in the hematopoietic tissue of the head kidney (Fig. 2d). Striped bass piscidin
229 4 was localized to mast cell progenitor cells of the gill arches (Fig. 2e) and hematopoietic cells in
230 hematopoietic tissue of the head kidney (Fig. 2f).
231 Striped bass piscidin 1 was detected in mast cell progenitor cells in the dorsal aorta (Fig.
232 3a) and in hematopoietic cells in the hematopoietic tissue of the kidney (Supplementary Fig. 2a)
233 of 19 dph larvae. Striped bass piscidin 3 was detected in the lamina propria of the mucosal
234 epithelium of the esophagus (Supplementary Fig. 2b), in mast cells migrating between the
235 columnar epithelial cells of the intestine (Fig. 3b), and in mast cell progenitor cells in the liver
236 and in the pancreatic epithelium (Fig. 3c). Striped bass piscidin 4 was localized to mast cells at
237 the base of the gill lamellae (Fig. 3d).
238 Striped bass piscidin 1 was not observed in any of the 21 dph fish sections. Striped bass
239 piscidin 3 staining was seen in cells within the mucosal epithelium of the thymus (Fig. 4a).
240 Striped bass piscidin 4 stained in mast cells in the vascular system of the primary gill lamellae
241 (Fig. 4b and Supplementary Fig. 3).
242 For the 27 dph larvae, striped bass piscidin 1 localized to mast cells in the lamina propria
243 underlying the stomach epithelium (Fig. 5a), striped bass piscidin 3 localized to mast cells in the
244 primary gill lamellae (Fig. 5b) and mast cells also in the lamina propria underlying the stomach
245 epithelium (Supplementary Fig. 4), and striped bass piscidin 4 localized to mast cells of the
246 primary gill lamellae (Fig. 5c) and the epidermis (Fig. 5d).
247 All three piscidin antibodies stained cells within the gill, stomach, anterior intestine, and
248 skin of 12 month old fish. For the gill, striped bass piscidin 1 was stained in an unknown cell

type in blood vessels in the pharyngeal teeth (Fig. 6a) and striped bass piscidin 3 (Fig. 6c) and striped bass piscidin 4 (Supplementary Fig. 5h and 5i) stained mast cells in the gill lamellae. Mast cells migrating through the columnar epithelium of the stomach were stained with the antibody against striped bass piscidins 1, 3, and 4 (Supplementary Fig. 5a, Supplementary Fig. 5e, and Fig. 6e, respectively). Striped bass piscidin 1 was found in mast cells in the submucosa and muscularis and in veins within the submucosa of the anterior intestine (Supplementary Fig. 5b and 5c). Striped bass piscidins 3 (Fig. 6d and Supplementary Fig. 5f) was found in mast cells within the lamina propria and mast cells migrating through the intestinal epithelium. Striped bass piscidin 4 (Fig. 6f and Supplementary Fig. 5j) was stained in mast cells located in the lamina propria and the intestinal epithelium. In the skin, striped bass piscidin 1 was stained in the stratum compactum of the dermis (Fig. 6b and Supplementary Fig. 5d), while both striped bass piscidins 3 (Supplementary Fig. 5g) and 4 (Supplementary Fig. 5k) were stained in mast cells in the epidermis.

The semi-quantitative rt-PCR failed to detect any piscidin gene expression in samples from 14 dph and earlier (*e.g.* post-vitellogenic oocytes, embryos, or larvae). The control gene *rpl9* produced a PCR product of the predicted size indicating that the reactions were successful.

4. DISCUSSION

This is the first description of tissue localization of multiple piscidins in the striped bass during its development. While piscidins appear to be localized to mast cells in the gills, stomach, intestine, and skin of adult fish, each piscidin studied here appears localized to hematopoietic, progenitor, and mature cell populations within different tissues of developing striped bass larvae

and fry. These differential staining patterns indicate that the piscidins are localized to similar cell types, but their production varies between tissue and maturation of cells.

Douglas et al. (2001) found the first evidence of pleurocidin transcripts in winter flounder (*Pleuronectes americanus*) at 13 dph, increasing thereafter during development. This coincides with the fact that we did not see any piscidin gene expression or immunofluorescent staining in larvae at 14 dph or earlier, but we did see staining at 16 dph and later. The only time point where piscidin 1 staining was not observed was at 21 dph, however the lack of staining observed in the tissue sections may have been due to lack of sufficient epitopes for recognition by the primary antibody. We conclude that the piscidins first appear as proteins sometime between 14 and 16 dph in striped bass fry and the gene transcripts likely are expressed concomitant to this as they are not detected in larvae earlier than 14 dph. The timing of this first appearance of piscidins is important, because striped bass fry are typically stocked into earthen ponds for fingerling production at 3-5 dph, after they have developed fully functional digestive organs (Harrell et al. 1990). These ponds also may be breeding grounds for pathogens not present in the hatchery. The ability to coordinate feeding resources, competition, and immune functions may be of the utmost importance during this critical time frame in the developing fish.

Piscine mast cells can be distinguished from acidophilic granulocytes by the presence of histamine in mast cells, but not acidophilic granulocytes (Mulero et al. 2008). It has previously been shown that the antibody used here specific for the striped bass piscidin 1 (anti-FFHH antibody, used in this study) immunostains mast cells, but not acidophilic granulocytes while the antibody specific for the striped bass piscidin 3 (anti-HAGR antibody, used in this study) immunostains both mast cells and acidophilic granulocytes in gilthead seabream (Mulero et al. 2008). Also, the antibody specific for striped bass piscidin 4 (anti-5.3 antibody, used in this

study) immunostains mast cells in the gill of striped bass, white bass, European sea bass, gilthead seabream, and red drum (*Sciaenops ocellatus*) (Corrales et al. 2010).

We observed immunofluorescent staining of piscidins in hematopoietic and immature mast cells or mast cell progenitor cells in the youngest fry (e.g., Fig. 2b, 2c, and 2d and Supplementary Fig. 1a and 1c) and mast cells in older fish (e.g., Fig. 6c, 6d, 6e, and 6f and Supplementary Fig. 5e, 5g, 5i, and 5k) in the developing hematopoietic and epithelial tissues. Hematopoietic cells have been observed in the head kidney of the gilthead seabream at 6 dph increasing in number thereafter (Mulero et al. 2007a). The appearance of mast cells in the older fry is expected as piscidins have been localized to mast cells in the subepithelial and connective tissues and mucosal epithelial tissues in adult fish. Silphaduang and Noga (2001) reported that mast cells in gill, skin, and gut, and those lining blood vessels in viscera, were piscidin-positive in adult hybrid striped bass. Also, pleurocidin was localized to mucin granules of skin and intestinal goblet cells in the winter flounder (*Pleuronectes americanus*) using transmission electron microscopy (Cole et al. 2000). Murray et al. (2003) localized pleurocidin gene expression and protein synthesis to eosinophilic granular cells, or mast cells, in the non-lamellar portion of the gill filament of the winter flounder. The staining of potential progenitor immune cells by antibodies against the piscidins as shown here could lead to the development of markers to follow the ontogeny of fish cells involved in immune processes.

Piscidins have been described as ubiquitous effectors of the innate immune system. Most previous studies have localized piscidin peptides to cells located within epithelial tissues where they can defend against pathogens. In this study, we show differential tissue and cellular distributions of three piscidins in the striped bass. Along with previous studies (Salger et al. 2016) we have shown that the different classes of piscidins may be specialized and that each of

these peptides may exhibit specific activities within a certain cell or tissue type and against different types of pathogens. This specialization also leads us to believe that through evolution of this peptide family the piscidins have become diversified to perform these specific functions. For example, the Class I piscidins (Salger et al. 2016), which includes striped bass piscidins 1 and 3 of this study have a greater activity against Gram-positive bacteria than either the Class II, which includes striped bass piscidin 4, or the Class III piscidins, whereas the Class II piscidins have greater activity against Gram-negative bacteria than either the Class I or Class III piscidins. Also, the Class III piscidins, while having little to no activity against bacteria, have potent activity against the ciliated protozoan, *Tetrahymena pyriformis* (Salger et al. 2016).

We observed striped bass piscidin 3 staining in mast cell progenitor cells located in the liver of 19 dph striped bass larvae. Mast cells were stained in the perch (*Perca fluviatilis*) liver during infection by *Triaenophorus nodulosus* with the same antibody used in this study (Dezfuli et al. 2014). AMPs have also been found in other cell types of the liver: the expression of cathelicidin-related antimicrobial peptide (CRAMP) has been demonstrated in murine macrophages (Rosenberger et al. 2004) and hepcidin in mouse Kupffer cells (Theurl et al. 2008).

This study is the first description of the tissue distribution and localization of three piscidins during striped bass development. Although no immunofluorescent staining was observed prior to age 16 dph, all three piscidins were observed in juvenile fish at every stage. These results demonstrate that the immune system is developing during this critical time and that the fish are acquiring the ability to fight infections. Knowing when piscidins are first expressed and in which cells and tissues is important for the survival of young striped bass at a critical time in their development.

5. ACKNOWLEDGEMENTS

The authors would like to acknowledge Craig V. Sullivan for kindly providing laboratory space to perform this work. This is publication number 109 from the North Carolina State University Pamlico Aquaculture Field Laboratory.

Funding: This work was supported by the National Sea Grant College Program, National Oceanic and Atmospheric Administration, to the North Carolina Sea Grant College Program [grant numbers NA16-RG-2251 (R/AF-43, R/AF-45 and R/MG-0510)]; the U.S.D.A. Agriculture and Food Research Initiative [grant number 2009-03571]; and the U.S.D.A. National Needs Graduate Fellowship [grant number 2007-38420-17763].

6. LITERATURE CITED

Abelli L, Pichietti S, Romano N, Mastrolia L, Scapiliati G. 1996. Immunocytochemical detection of thymocyte antigenic determinants in developing lymphoid organs of sea bass *Dicentrarchus labrax* (L.). *Fish and Shellfish Immunology* 6:493-505.

Bulet P, Stöcklin R, Menin L. 2004. Anti-microbial peptides: from invertebrates to vertebrates. *Immunological Reviews 2004* 198:169-184.

Chapman RW, Reading BJ, Sullivan CV. 2014. Ovary transcriptome profiling via artificial intelligence reveals a transcriptomic fingerprint predicting egg quality in striped bass, *Morone saxatilis*. *PLoS ONE* 9:e96818.

Cole AM, Darouiche RO, Legarda D, Connell N, Diamond G. 2000. Characterization of a fish antimicrobial peptide: gene expression, subcellular localization, and spectrum of activity. *Antimicrobial Agents and Chemotherapy* 44:2039-2045.

Colorni A, Ullal A, Heinisch G, Noga EJ. 2008. Activity of the antimicrobial polypeptide piscidin 2 against fish ectoparasites. *Journal of Fish Diseases* 31:423-432.

Corrales J, Mulero I, Mulero V, Noga EJ. 2010. Detection of antimicrobial peptides related to piscidin 4 in important aquacultured fish. *Developmental and Comparative Immunology* 34:331-343.

Dezfuli BS, Giari L, Lorenzoni M, Manera M, Noga EJ. 2014. Perch liver reaction to *Triaenophorus nodulosus* plerocercoids with an emphasis on piscidins 3, 4 and proliferative cell nuclear antigen (PCNA) expression. *Veterinary Parasitology* 200:104-110.

Diamond G, Beckloff N, Weinberg A, Kisich KO. 2009. The roles of antimicrobial peptides in innate host defense. *Current Pharmaceutical Design* 15:2377-2392.

dos Santos NM, Romano N, de Sousa M, Ellis AE, Rombout JH. 2000. Ontogeny of B and T cells in sea bass (*Dicentrarchus labrax*, L.). *Fish and Shellfish Immunology* 10:583-596.

Douglas SE, Gallant JW, Gong Z, Hew C. 2001. Cloning and developmental expression of a family of pleurocidin-like antimicrobial peptides from winter founder, *Pleuronectes americanus* (Walbaum). *Developmental and Comparative Immunology* 25:137-147.

Dungan CF, Roberson BS. 1993. Binding specificities of mono- and polyclonal antibodies to the protozoan oyster pathogen *Perkinsus marinus*. *Diseases of Aquatic Organisms* 15:9-22.

Hanif A, Bakopoulos V, Dimitriadis GJ. 2004. Maternal transfer of humoral specific and non-specific immune parameters to sea bream (*Sparus aurata*) larvae. *Fish and Shellfish Immunology* 17:411-435.

Harrell RM, Kerby JH, Minton RV. 1990. Culture and propagation of striped bass and its hybrids. American Fisheries Society, Bethesda, MD.

Hayman JR, Lobb CJ. 1993. Immunoglobulin in the eggs of the channel catfish (*Ictalurus punctatus*). *Developmental and Comparative Immunology* 17:241-248.

Huttenhuis HBT, Grou CPO, Taverne-Thiele AJ, Taverne N, Rombout JHWM. 2006. Carp (*Cyprinus carpio* L.) innate immune factors are present before hatching. *Fish and Shellfish Immunology* 20:586-596.

Løvoll M, Kilvik T, Boshra H, Bøgwald J, Sunyer JO, Dalmo RA. 2006. Maternal transfer of complement components C3-1, C3-3, C3-4, C4, C5, C7, Bf, and Df to offspring in rainbow trout (*Oncorhynchus mykiss*). *Immunogenetics* 58:168-179.

Mulero I, Chaves-Pozo E, García-Alcázan A, Meseguer J, Mulero V, García-Ayala A. 2007a. Distribution of the professional phagocytic granulocytes of the bony fish gilthead seabream (*Sparus aurata* L.) during the ontogeny of lymphomyeloid organs and pathogen entry sites. *Developmental and Comparative Immunology* 31:1024-1033.

Mulero I, García-Ayala A, Meseguer J, Mulero V. 2007b. Maternal transfer of immunity and ontogeny of autologous immunocompetence of fish: A minireview. *Aquaculture* 268:244-250.

Mulero I, Noga EJ, Meseguer J, García-Ayala A, Mulero V. 2008. The antimicrobial peptides piscidins are stored in the granules of professional phagocytic granulocytes of fish and are delivered to the bacteria-containing phagosome upon phagocytosis. *Developmental and Comparative Immunology* 32:1531-1538.

Mumford S, Heidel J, Smith C, Morrison J, MacConnell B, Blazer V. 2007. Fish Histology and Histopathology; United States Fish and Wildlife Service, National Conservation Training Center.

Murray HM, Gallant JW, Douglas SE. 2003. Cellular localization of pleurocidin gene expression and synthesis in winter flounder gill using immunohistochemistry and *in situ* hybridization. *Cell and Tissue Research* 312:197-202.

Pálffy R, Gardlík R, Behuliak M, Kadasi L, Turna J, Celec P. 2009. On the physiology and pathophysiology of antimicrobial peptides. *Molecular Medicine* 15:51-59.

Picchietti S, Abelli L, Buonocore F, Randelli E, Fausto AM, Scapigliati G, Mazzini M. 2006. Immunoglobulin protein and gene transcripts in sea bream (*Sparus aurata* L.) oocytes. *Fish and Shellfish Immunology* 20:398-404.

Quesada J, Villena MI, Navarro V. 1994. Ontogeny of the sea bass spleen (*Dicentrarchus labrax*): a light and electron microscopic study. *Journal of Morphology* 221:161–176.

Reading BJ, Hiramatsu N, Schilling J, Molloy KT, Glassbrook N, Mizuta H, Luo W, Baltzegar DA, Williams VN, Todo T, Hara A, Sullivan SV. 2014. Lrp13 is a novel vertebrate lipoprotein receptor that binds vitellogenins in teleost fishes. *Journal of Lipid Research* 55:2287-2295.

Rosenberger CM, Gallo RL, Finlay BB. 2004. Interplay between antibacterial effectors: A macrophage antimicrobial peptide impairs intracellular Salmonella replication. *Proceedings of the National Academy of Sciences* 101:2422-2427.

Ruangsri J, Salger SA, Caipang CMA, Kiron V, Fernandes JMO. 2012. Differential expression and biological activity of two piscidin paralogues and a novel splice variant in Atlantic cod (*Gadus morhua* L.). *Fish and Shellfish Immunology* 32:396-406.

Salger SA, Cassady KR, Reading BJ, Noga EJ. 2016. A diverse family of host-defense peptides (piscidins) exhibit specialized anti-bacterial and anti-protozoal activities in fishes. *PLoS ONE* 11:e0159423.

461 Salger SA, Reading BJ, Baltzegar DA, Sullivan CV, Noga EJ. 2011. Molecular characterization
462 of two isoforms of piscidin 4 from the hybrid striped bass (*Morone chrysops* x *Morone*
463 *saxatilis*). *Fish and Shellfish Immunology* 30:420-424.
464
465 Scapigliati G, Romano N, Buonocore F, Picchietti S, Baldassini MR, Prugnoli D, Galice A,
466 Meloni S, Secombes CJ, Mazzini M, Abelli L. 2002. The immune system of sea bass,
467 *Dicentrarchus labrax*, reared in aquaculture. *Developmental and Comparative*
468 *Immunology* 26:151-160.
469
470 Schroder MB, Villena AJ, Jørgensen TØ. 1998. Ontogeny of lymphoid organs and
471 immunoglobulin producing cells in Atlantic cod (*Gadus morhua* L.). *Developmental and*
472 *Comparative Immunology* 22:507–517.
473
474 Seppola M, Johnsen H, Mennen S, Myrnes B, Tveiten H. 2009. Maternal transfer and
475 transcriptional onset of immune genes during ontogenesis in Atlantic cod. *Developmental*
476 *and Comparative Immunology* 33:1205-1211.
477
478 Silphaduang U, Noga EJ. 2001. Peptide antibiotics in mast cells of fish. *Nature* 414:268-269.
479
480 Silphaduang U, Colorni A, Noga EJ. 2006. Evidence for widespread distribution of piscidin
481 antimicrobial peptides in teleost fish. *Diseases of Aquatic Organisms* 72:241-252.
482
483 Theurl M, Theurl I, Hochegger K, Obrist P, Subramaniam N, van Rooijen N, Schuemann K,
484 Weiss G. 2008. Kupffer cells modulate iron homeostasis in mice via regulation of
485 hepcidin expression. *Journal of Molecular Medicine* 86:825-835.
486
487 Valore EV, Park CH, Quayle AJ, Wiles KR, McCray PB, Ganz T. 1998. Human β-defensin-1: an
488 antimicrobial peptide of urogenital tissues. *Journal of Clinical Investigations* 101:1633-
489 1642.
490
491 Wang Z, Zhang S, Wang G, An Y. 2008. Complement activity in the egg cytosol of zebrafish
492 *Danio rerio*: evidence for the defense role of maternal complement components. *PLoS*
493 *ONE* 3:e1463.
494
495 Williams VN, Reading BJ, Amano H, Hiramatsu N, Schilling J, Salger SA, Williams TI, Gross
496 K, Sullivan SV. 2014. Proportional accumulation of yolk proteins derived from multiple
497 vitellogenins is precisely regulated during vitellogenesis in striped bass (*Morone*
498 *saxatilis*). *Journal of Experimental Zoology* 321A:301-315.
499
500 Zapata A, Diez B, Cejalvo T, Gutiérrez-de Frías C, Cortés A. 2006. Ontogeny of the immune
501 system of fish. *Fish and Shellfish Immunology* 20:126-136.
502
503
504
505
506

Figure 1. 16 dph striped bass larvae. Hematoxylin and eosin (H&E) stain. B = buccal cavity, D = dorsal aorta, Es = esophagus, HK = head kidney, I = anterior intestine, Li = liver, N = notochord, TK = trunk kidney. Scale bar = 200 μm.

Figure 2. Immunostaining of 16 dph striped bass larvae with the anti-FFHH (striped bass piscidin 1) antibody (green; a, b), the anti-HAGR (striped bass piscidin 3) antibody (green; c, d), and the anti-5.3 (striped bass piscidin 4) antibody (green; e, f). Staining of striped bass piscidin 1 was localized to **(a)** mast cell progenitor cells (arrowheads) located in the buccal cavity and chromatin counterstained with Hoechst 44432 (blue) and **(b)** hematopoietic cells (arrows) in hematopoietic tissue of the head kidney. Striped bass piscidin 3 was found in **(c)** mast cell progenitor cells (arrowheads) in the dorsal aorta and **(d)** hematopoietic cells in hematopoietic tissue of the head kidney. Striped bass piscidin 4 was localized to **(e)** mast cell progenitor cells (arrowheads) in the gill arches and **(f)** hematopoietic cells (arrows) in hematopoietic tissue of the head kidney and chromatin counterstained with Hoechst 44432 (blue). (a, f) Scale bar = 20 μm. (b, e) Scale bars = 100 μm. (c, d) Scale bars = 50 μm.

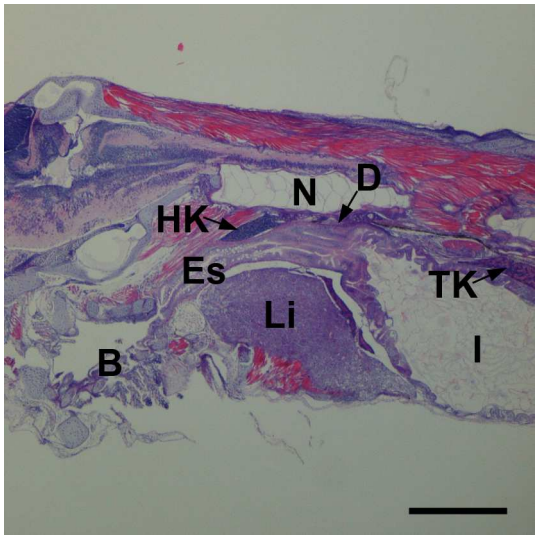
Figure 3. Immunostaining of 19 dph striped bass larvae with the anti-FFHH (striped bass piscidin 1) antibody (green; a, b), the anti-HAGR (striped bass piscidin 3) antibody (green; c-e), and the anti-5.3 (striped bass piscidin 4) antibody (green; f). Staining of striped bass piscidin 1 was localized **(a)** to mast cell progenitor cells in the dorsal aorta. Striped bass piscidin 3 was found in **(b)** mast cells (arrowheads) migrating between the columnar epithelial cells of the intestine and **(c)** mast cell progenitor cells in the liver and pancreatic epithelium (lower left inset is higher magnification of liver showing stained cells). Striped bass piscidin 4 **(d)** was localized to mast cells (arrowheads) at the base of the gill lamellae. Upper right inset of b and c show negative controls (excluding the primary antibody) from the immunostaining procedure. Chromatin was counterstained with Hoechst 44432 (blue) in b and c (inset). L = anterior intestine lumen, Li = liver, N = notochord, P = pancreas. (a, d) Scale bars = 100 μm. (b) Scale bar = 500 μm. (c) Scale bars = 200 μm.

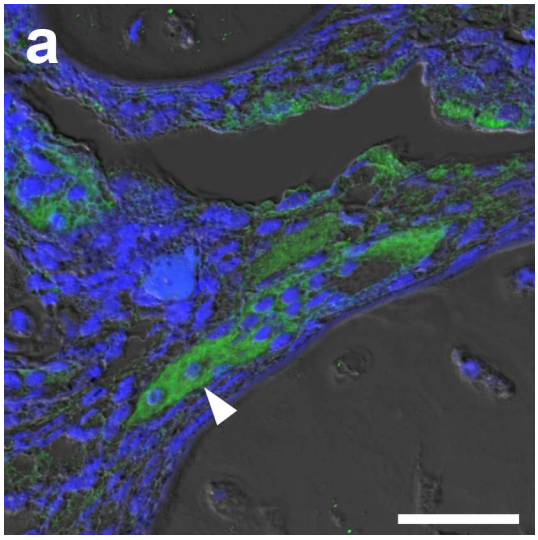
Figure 4. Immunostaining of 21 dph striped bass fry with the anti-HAGR (striped bass piscidin 3) antibody (green; a) and the anti-5.3 (striped bass piscidin 4) antibody (green; b). **(a)** Striped bass piscidin 3 was localized to cells (arrows) within the mucosal epithelium of the thymus. Striped bass piscidin 4 was localized to **(b)** mast cells in the vascular system of the primary gill lamellae (arrowheads). Upper right inset of a shows the negative control (excluding the primary antibody) from the immunostaining procedure. B = buccal cavity, G = gills, T = thymus. (a) Scale bar = 200 μm. (b) Scale bar = 100 μm.

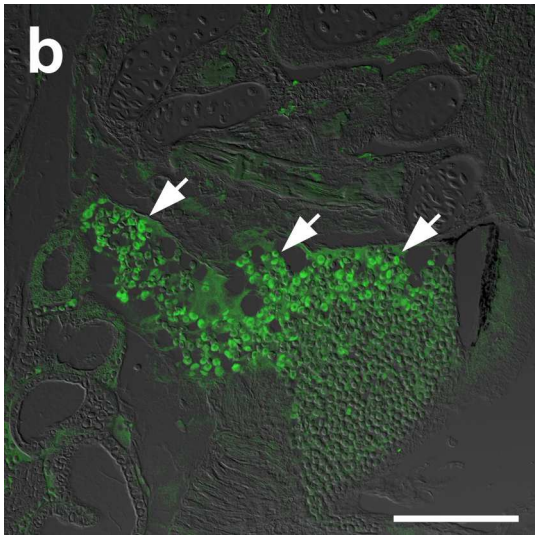
Figure 5. Immunostaining of 27 dph striped bass fry with the anti-FFHH (striped bass piscidin 1) antibody (green; a), the anti-HAGR (striped bass piscidin 3) antibody (green; b, c), and the anti-5.3 (striped bass piscidin 4) antibody (green; d, e). **(a)** Striped bass piscidin 1 was stained in mast cells (arrowheads) of the submucosa underlying the stomach epithelium. **(b)** Striped bass piscidin 3 was localized to mast cells in the primary gill lamellae. Striped bass piscidin 4 was

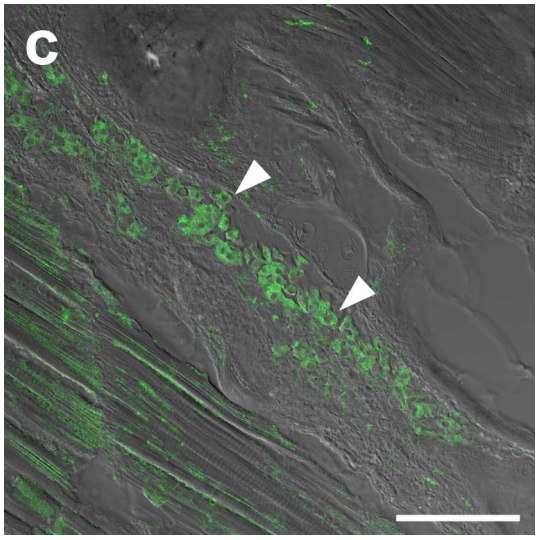
stained in mast cells (arrowheads) in the **(c)** primary gill lamellae and **(d)** epidermis. E = stomach epithelium, Ep = epidermis, L = stomach lumen, S = submucosa. (a) Scale bar = 100 μm. (b-d) Scale bar = 50 μm.

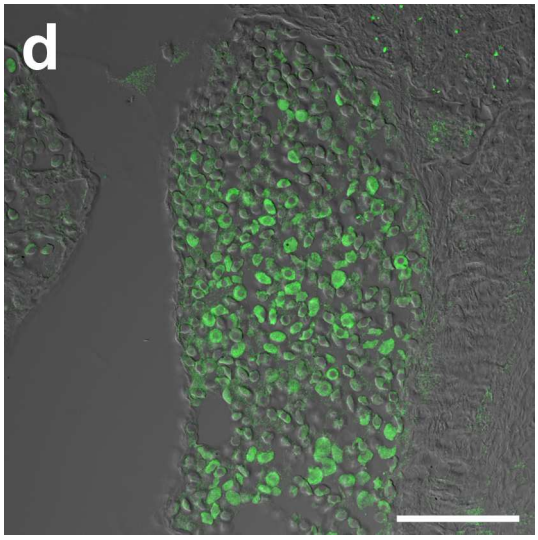
Figure 6. Immunostaining of 12 mo old striped bass tissues with the anti-FFHH (striped bass piscidin 1) antibody (green; a), the anti-HAGR (striped bass piscidin 3) antibody (green; b), and the anti-5.3 (striped bass piscidin 4) antibody (green; c-d). Striped bass piscidin 1 was localized to **(a)** cells (arrowheads) in blood vessels in the pharyngeal teeth and **(b)** in the stratum compactum of the dermis (arrows). Striped bass piscidins 3 was localized to **(c)** mast cells (arrows) in the gill lamellae and **(d)** mast cells (arrowheads) within the lamina propria and those migrating through the intestinal epithelium. Striped bass piscidin 4 antibodies stained **(e)** mast cells (arrowheads) migrating through the columnar epithelium of the stomach and **(f)** mast cells (arrowheads) located in the lamina propria and the intestinal epithelium. Chromatin is counterstained with Hoechst 44432 (blue) in a, c-f. E = epithelium. (a, d-f) Scale bar = 50 μm. (b) Scale bar = 100 μm. (c) Scale bar = 20 μm.

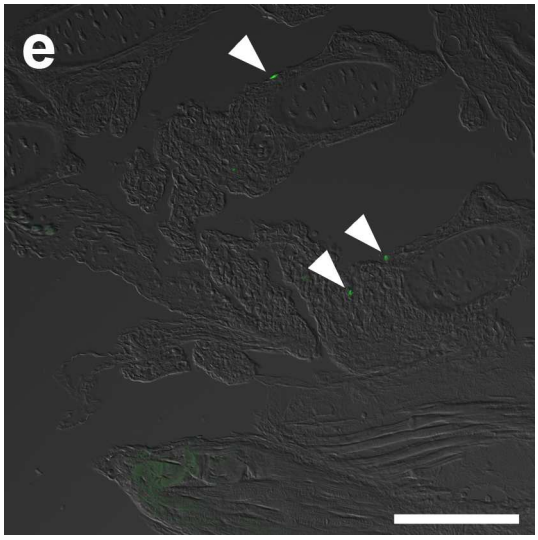

N
D
HK
Es
TK
Li
I
B

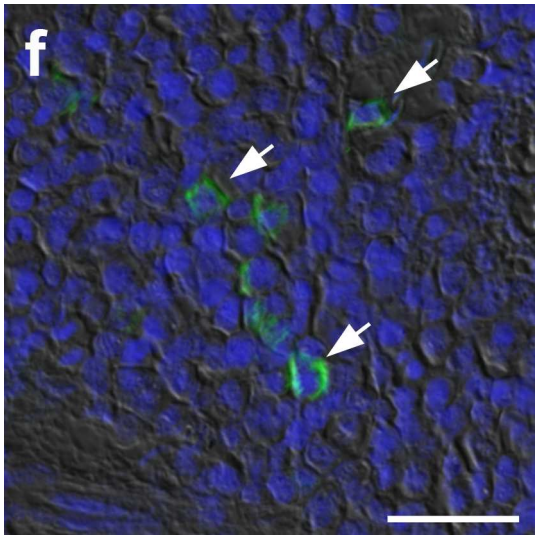

a

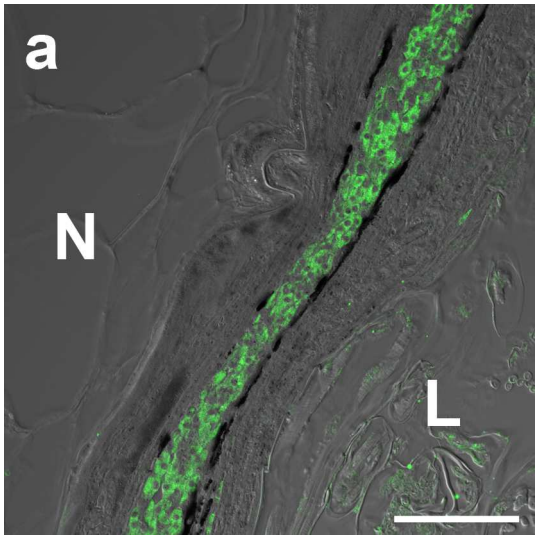

b

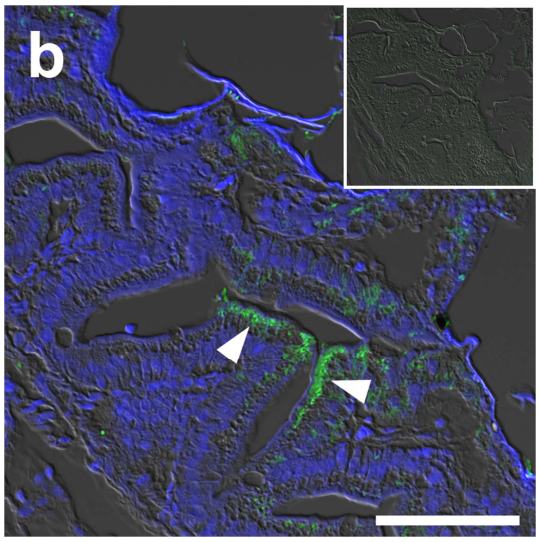

C

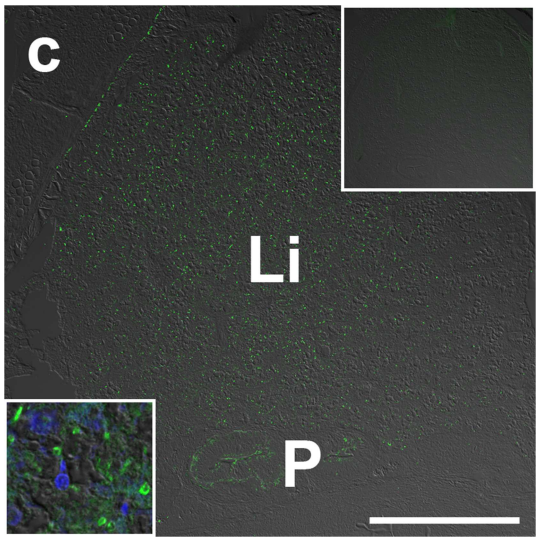

d

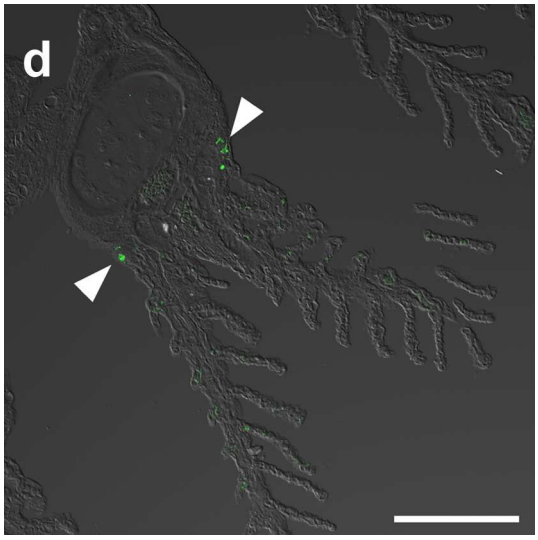

e

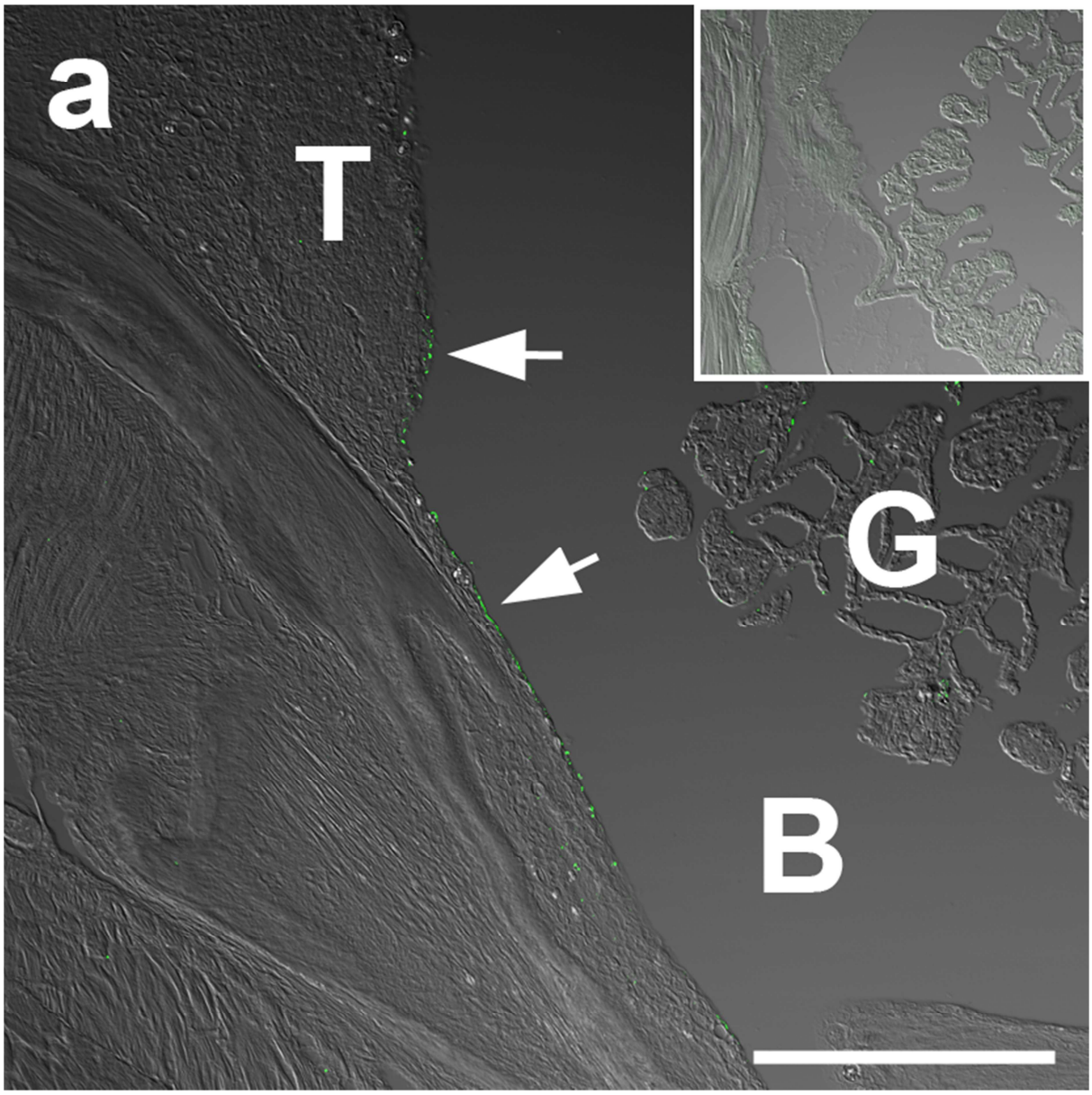

f

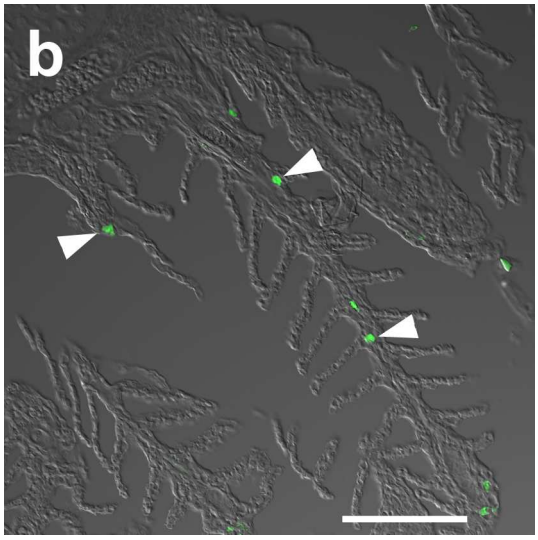

a
N
L

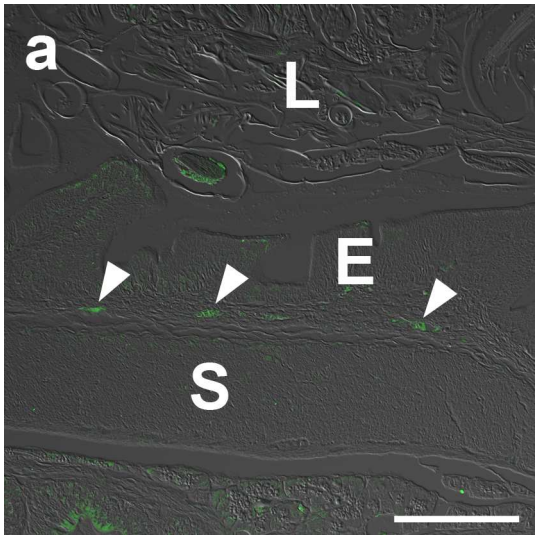

b

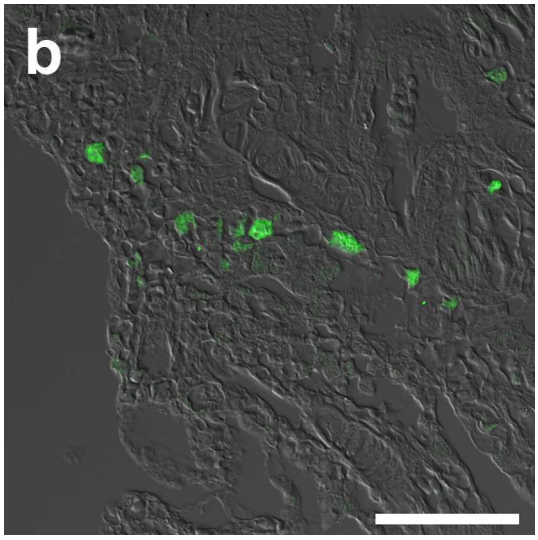

C
Li
P

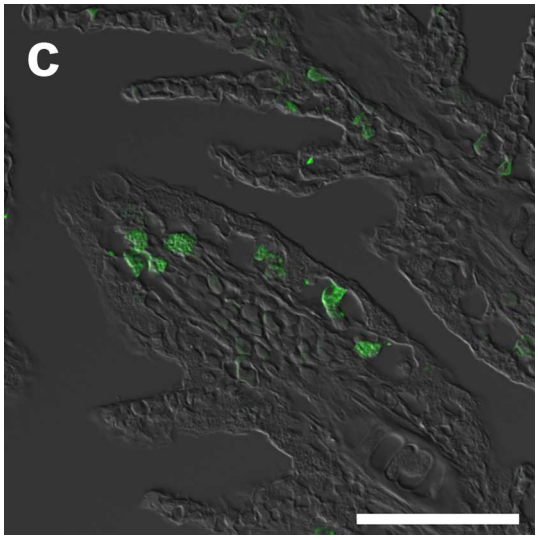

d

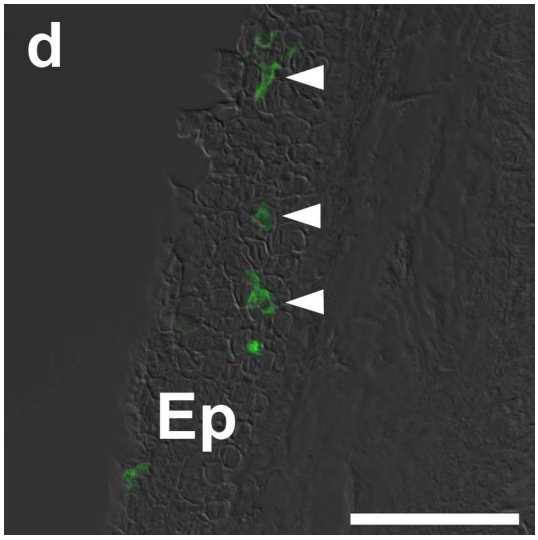

a
T
G
B

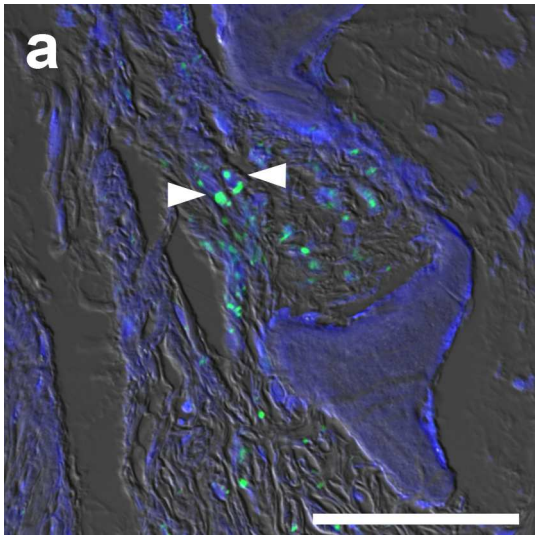

b

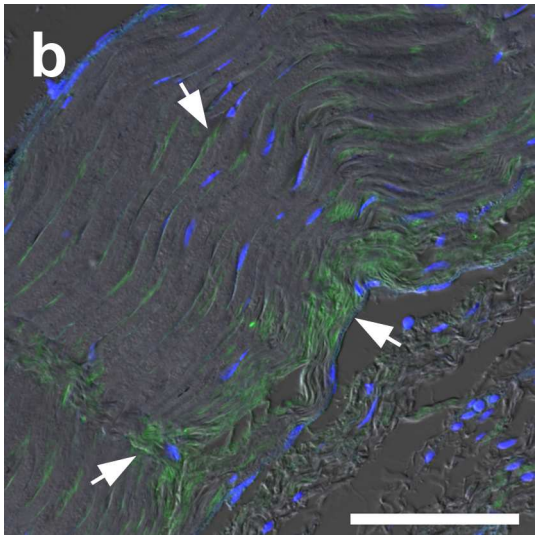

a
L
E
S

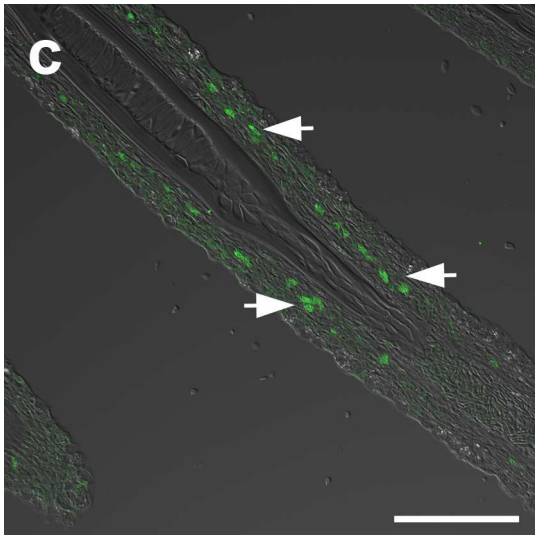

b

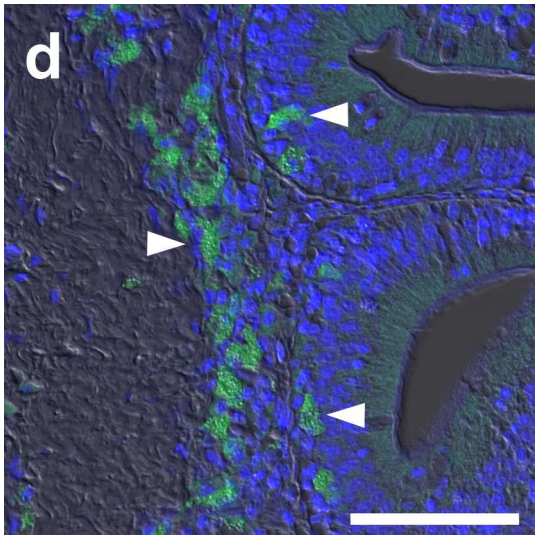

c

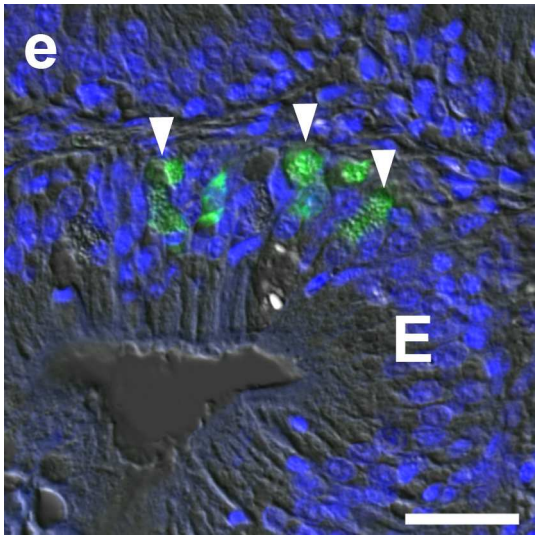

d
Ep

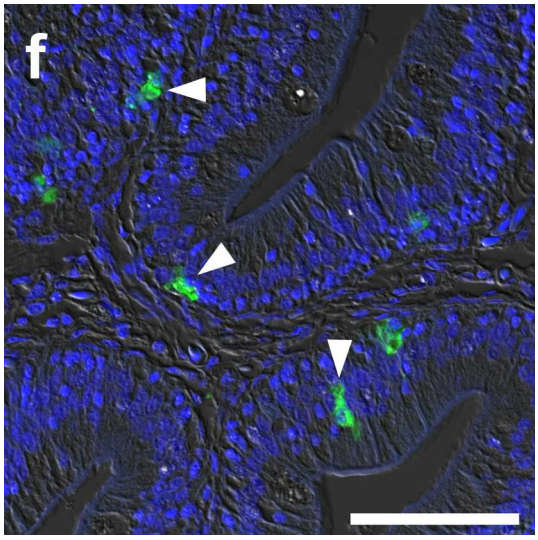

a


b


C


d


e
E


f

Highlights:

Manuscript Reference Number: FSIM-D-16-00690

Tissue Localization of Piscidin Host-Defense Peptides during Striped Bass (*Morone saxatilis*) Development

Scott A. Salger, Benjamin J. Reading, Edward J. Noga

- The ontogeny of the host-defense peptides, piscidins, was investigated
- Piscidins first appear in the striped bass between 14 and 16 dph
- Piscidins showed variable cellular and tissue staining patterns
- Could relate to differences in tissue susceptibility or pathogen specificity
- Supports observations that each piscidin has different (specialized) functions