2019

ONTOGENY OF THE LATERAL LINE AND VISUAL SYSTEMS OF A CARIBBEAN REEF GOBY, *ELACATINUS LORI*

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ONTOGENY OF THE LATERAL LINE AND VISUAL SYSTEMS OF A
CARIBBEAN REEF GOBY, *ELACATINUS LORI*

BY

KATIE R. NICKLES

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE DEGREE OF

MASTER OF SCIENCE

IN

BIOLOGICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2019

MASTER OF SCIENCE THESIS
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2019

ABSTRACT

Chapter 1

Gobies (Family Gobiidae) have a complex mechanosensory lateral line system composed of reduced cranial and trunk canals and a proliferation of small superficial neuromasts, perched on the tips of “sensory papillae”, which occur in several linear series on the head, trunk and tail. *Elacatinus lori* is being used as a model species for larval orientation and dispersal and the ability to rear it in the lab has allowed the use of a suite of morphological methods to provide the first description of the post-embryonic development of the lateral line system from hatch through settlement and to adulthood for any goby species. The distribution of canal neuromasts (CNs) and superficial neuromasts (SNs) was described in adult *E. lori* using fluorescent imaging, revealing six cranial superficial neuromast series composed of 33 lines of neuromasts, reduced cranial lateral line canals (only the supraorbital, postotic, and preopercular canals are present), 31 lines on the trunk and three lines on the caudal fin. The total number of SNs on the head increases linearly through the larval period to settlement (at 35 dph) while all CNs are present on the surface of the skin by about 15 dph before becoming enclosed in canals. Both CNs and SNs are diamond-shaped at hatch, which along with the proliferation of neuromasts within lines may have interesting implications for the sensitivity and functional role of these neuromasts, especially during the larval period. This study also compares the adult lateral line morphology of *E. lori* to congeners and representatives of the sister genus, *Tigrigobius*. We found that among *Elacatinus* and *Tigrigobius* species there are significant correlations between SN number and microhabitat, which indicates

small-scale environmental factors, like a sponge-dwelling habit versus a coral-dwelling habit, can have significant impacts on lateral line system morphology.

Chapter 2

For many years, researchers have been trying to determine how pelagic larvae navigate the water column and find settlement sites, but this still remains unclear.

Elacatinus lori, an obligate sponge-dwelling goby native to Belizean reefs, is a model species for larval coral reef fish navigation and dispersal. This study looks at the ontogeny of the visual system (opsin gene expression and eye size and structure) using both morphological and genomic analyses to infer larval sensory capabilities that are likely to play a role in larval orientation and settlement site selection. Results showed that the morphology of the eye and retina in *E. lori* is very similar to those in other fish species studied. Some variation was found in the growth rate of the different retinal layers. The molecular portion of the study faced some challenges in that not all opsin sequences could be identified and so a gene expression analysis could not be completed. Alternative methods for future analysis of opsin expression in *E. lori* are presented and whole genome sequencing and transcriptomics are suggested as methods that will need to be used to identify all other opsin sequences.

ACKNOWLEDGEMENTS

We thank our field research team (C. Burns, K. Catalano, R. Chaput, C. D'Aloia, J. Ferrito, M. Foretich, R. Francis, F. Francisco, E. Schlatter, and D. Scolaro) who helped to collect and rear gobies in Belize, and the staff at the International Zoological Expeditions and Wee Wee Caye field stations for logistical support and for the use of their facilities. We thank Louie Kerr and his staff at the Central Microscopy Facility at the MBL (Woods Hole) for their SEM expertise. We thank Andrew Williston (MCZ) for his μ CT expertise, Zac Tressin for help with μ CT reconstructions, Alec Mauk for help with histology data collection, and Aubree Jones and Elizabeth Molnar for help with SEM data collection and for reading earlier versions of the manuscript. We would like to thank Dr. Christopher Lane and his lab, specifically Jillian Freese and Elizabeth Hunter, for their molecular supplies and expertise. A portion of this material is based upon work conducted at a Rhode Island NSF EPSCoR research facility, the Genomics and Sequencing Center, supported in part by NSF EPSCoR Cooperative Agreement #EPS-1004057. Funded by NSF grant #1459546 to JFW, and NSF #1459546 to PMB.

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CHAPTER 1

INTTODUCTION

Sensing and responding appropriately to environmental cues is a challenge that all organisms face. As the first vertebrates, fishes evolved several sensory modalities to obtain information from their aquatic environments. In order to understand these sensory systems, questions must be asked about how the sensory systems function and develop.

The mechanosensory lateral line system, present in all fishes (and some amphibians), allows them to detect directional and low frequency water flows within a distance of a few body lengths. These hydrodynamic stimuli are used for prey detection, predator avoidance, orientation, schooling and communication (Montgomery et al., 2014; Webb, 2014b; Butler & Maruska, 2016). The lateral line system is composed of sensory receptor organs (neuromasts) that are distributed over the head and the body either on the skin (superficial neuromasts, SNs) or enclosed in pored, bony canals (canal neuromasts, CNs; Figure 1.1C). Neuromasts are comprised of bundles of ciliated sensory hair cells located in a sensory strip surrounded by non-sensory support cells (Figure 1.1A). The ciliary bundle on the apical surface of each hair cell is made up of many stereocilia in ascending gradation adjacent to the kinocilia, which is much longer than the stereocilia (Flock & Duvall, 1965). This morphological polarity defines the physiological polarization (the axis of best physiological sensitivity to water flows) of each hair cell. The hair cells within a neuromast are oriented 180° to each other, defining a single axis of best physiological sensitivity (Figure 1.1B). The ciliary bundles extend into the gelatinous cupula, whose base is the same shape as the neuromast (Figure 1.1). The cupula extends into the external environment and is deflected by water flows that bend

the kinocilia away from or toward the stereocilia in the hair cells. This bending triggers a response from the hair cells that is transmitted to the afferent neurons innervating the hair cells (Kindt et al., 2012). Stimuli are transmitted via the anterior, middle, or posterior lateral line nerves, depending on location on the head or body, to the octavolateralis area in the rhombencephalon (medulla oblongata; Wullimann & Grothe, 2014). Canal neuromasts in pored canals function as accelerometers while SNs on the skin function as velocimeters, allowing fish to extract different types of sensory information from water flows in the surrounding environment (Denton & Gray, 1989; McHenry & Liao, 2014).

The lateral line system of bony fishes, and of actinopterygians in particular, is composed of CNs located in the epithelial lining of the hollow, cylindrical lateral line canals, at locations between sequential canal pores. The number of CNs and pores in a given canal may vary among species, but the subset of bones in which the canals are found is conserved in all bony fishes (Figure 1.1D). The cranial lateral line canals of bony fishes are: the supraorbital canal (in the nasal and frontal bones), the infraorbital canal (in the lacrimal and other infraorbital bones), the preopercular canal (in the preoperculum), the mandibular canal (in the dentary and anguloarticular), the otic canal (in the pterotic), the supratemporal commissure (in the lateral and medial extracapsular bones), and the postotic canal (in the post-temporal bone). In addition, a trunk canal in a series of overlapping lateral line scales, which runs from the posterior margin of the skull to the caudal peduncle, is typically present (Webb, 2014b).

Among species of fishes, the distribution of SNs on the head, trunk and tail is more variable than that of the CNs, which are restricted to canals. Some of the more common distribution patterns of SNs include SN lines on the head (e.g., salmon; Jollie,

1984), SNs located adjacent to canals as accessory SNs (e.g., cichlids; Becker et al., 2016), SNs in dense fields on the head (e.g., tetras - Molnar et al., unpub. data; giant danio [*Devario aequipinnatus*] - Mekdara et al., 2018), or as SN lines that represent the CNs of canals that do not form (e.g., “replacement NMs” in gobies, Wongrat & Miller, 1991 and Webb, 1989a).

The lateral line canals are variable in structure (e.g., narrow, widened, reduced, branched) and neuromasts vary with respect to shape, size and distribution among taxa (Webb, 2014b). Differences in canal morphology and the relative number of SNs tend to be correlated with the environmental conditions in which a species lives. For example, fishes that live in high flow environments (e.g. salmonids in freshwater streams and rivers) tend to have well ossified canals and few SNs (Jollie, 1984; Engelmann et al., 2002; Jones & Webb, unpub. data). Whereas fishes living in low flow environments, like ponds, underwater caves, or the deep sea, tend to have cranial canals that are widened or reduced and often exhibit an extreme proliferation of SNs (e.g. Asaoka et al., 2012; Montgomery et al., 2001; Marranzino & Webb, 2018).

The development of the lateral line system has been well studied in only a few species of fishes (e.g. zebrafish, Webb & Shirey, 2003; Ghysen & Dambly-Chaudiere, 2004; cichlids, Webb, 1989b; Becker et al., 2016). Although adult lateral line morphology may be diverse, all fishes that have been studied appear to follow the same basic developmental trends. All neuromasts appear in the epidermis as small, round SNs, each with only a few hair cells (Webb, 2014a). As a fish develops, neuromasts that are destined to become enclosed in a canal (presumptive CNs) will begin to sink into a groove as the ridges of the bony canal walls grow up on either side and fuse over the

neuromast, resulting in a neuromast fully enclosed in a hollow, fluid-filled canal tube (Tarby & Webb 2003; Bird & Webb 2014). Once enclosed in a bony canal, CN number remains constant (Becker et al., 2016). In contrast, SNs, which remain on the surface of the skin, are not limited by canal enclosure and may continue to proliferate into lines, fields, or clusters throughout the larval and juvenile stages (Becker et al., 2016).

The Gobiiformes is a speciose order of percomorph fishes with over 2,000 species (Patzner et al., 2011) that have radiated into an incredibly diverse range of marine and freshwater habitats. Gobiiform fishes, and those in the Family Gobiidae (the gobies) in particular, have an interesting modification of their lateral line system that is characterized by a reduction of the cranial and trunk canals and a dramatic proliferation of SNs on their head, trunk and tail (e.g., Sanzo, 1911; Wongrat & Miller, 1991, Asaoka et al., 2012). The SNs, which can number in the thousands (Asaoka et al., 2012; Sanzo, 1911), typically sit on “sensory papillae”, which are small finger-like extensions of the skin. In some gobies, these papillae extend the SNs and their cupulae well beyond the mucus layer covering the skin, which presumably increases their exposure and thus their sensitivity to water flows (Marshall, 1986).

Early studies that examined the patterning of the SNs (sensory papillae) in gobies include Sanzo (1911), which was the first study to provide a universal system for naming the canal pores and SN lines. The supraorbital, postotic, and a portion of the preopercular canals are the only canals commonly present in gobies. Sanzo (1911) described nine series or groups of SNs that occur in lines on the head, trunk and tail (Figure 1.2A). Among species, the lines of SNs on the head occur in two distinct patterns: “transverse” where the SNs ventral to the eye are in rostral-caudal lines and “longitudinal” where the

SNs ventral to the eye are in dorsal-ventral lines (radiating away from the orbit; Figure 1.2; Miller et al., 1980). This naming system was expanded by Wongrat and Miller (1991) who increased the number of species described and also added descriptions of the innervation patterns of these SNs.

An increasing number of studies have looked at the lateral line system in the adults of a diversity of gobiiform species (e.g. - Akihito, 1986; Asaoka et al., 2012; Asaoka et al., 2014). The most primitive extant gobioid (*Rhyacichthys aspro*), the outgroup to other gobiiforms, has 227 CNs and 308 SNs (on one side of the head, body and tail; Asaoka et al., 2014), while a goby with an extreme SN proliferation (*Glossogobius olivaceus*) has only 9 CNs (its canals are partially reduced, leaving others as CNs homologs on the skin) and 4828 SNs (on one side of the head, trunk and tail; Asaoka et al., 2012). Marshall (1986) was the first to look at goby neuromasts using scanning electron microscopy. He showed that the SNs in *Gobius niger* are located on the tips of sensory papillae and have an elongate diamond shape with a central sensory strip perpendicular to the long axis of the SN. This study showed that all SN lines in *Gobius niger* have the same SN arrangement and orientation, except the medial line of the pair of SN lines on the mandible (line “i” in Figure 1.2A). Marshall (1986) hypothesized that this medial mandibular line may represent homologs of the CNs of the mandibular canal, retaining the directional of CNs even though they were no longer enclosed in canals.

Only a few studies have looked at any aspect of the ontogeny of gobies or of their lateral line systems (e.g. Konagai & Rimmer, 1984; Ahnelt & Scattolin, 2003). However, they either did not examine the entire ontogeny through the adult stage or they only mentioned the structure of the lateral line system incidentally in general descriptions of

ontogeny. Given the speciose nature of the gobiiform fishes, it is not surprising that only a small fraction of extant species has been studied in any detail.

Elacatinus lori, a Caribbean reef goby, can be reared in the lab, which has facilitated its use as a model for larval dispersal (Majoris et al., 2018a, b) and the potential role of the various sensory systems in larval behavior (olfactory and gustatory systems, Hu et al., 2018; auditory system, Hu et al, in prep). *Elacatinus* (neon gobies) and *Tigrigobius* (its sister genus) are members of the North American seven-spined gobies (Family Gobiidae, Tribe Gobiosomatini, Thacker & Roje, 2011). *Elacatinus* is composed of 21 tropical marine species found on coral reefs, which all have a bright longitudinal stripe, a rostral frenum (connection between the snout and upper lip) and generally lack scales. *Tigrigobius* is composed of 12 tropical coral reef species that tend to be brightly colored (Van Tassell, 2011). *Elacatinus* includes sponge-dwelling planktivores and coral-dwelling cleaners, and these ecological specializations are correlated with their phylogenetic placement. *Elacatinus* is comprised of three clades: a basal Pacific species (a monotypic clade), the sponge-dwelling planktivores, and the coral-dwelling cleaners (Figure 1.3, Ruber et al., 2003; Taylor & Hellberg, 2005). *Tigrigobius* is comprised of species that occupy a more diverse set of microhabitats including coral-dwelling cleaners, sponge-dwellers, chiton burrowers, and urchin associated species. The phylogeny of *Tigrigobius* is less clear, as it may not be a monophyletic group (Ruber et al., 2003; Taylor & Hellberg, 2005). Neither the lateral line system of *Elacatinus* nor *Tigrigobius* has been studied in detail, but some aspects of the lateral line system have been used as a morphological character in a few instances (Figure 1.2, *E. oceanops* and *T. macrodon*, Miller, 1972; *T. limbaughi*, Hoese & Reader, 2001). The ability to rear *E. lori* in the lab

and some data on the lateral line system of other *Elacatinus* and *Tigrigobius* species provide a useful context in which to explore the comparative morphology of the lateral line system in this group of fishes.

This study aims to answer three questions: 1) What is the adult morphology of the lateral line system in *E. lori*? 2) How does the complex proliferation of superficial neuromast lines develop during the larval stage and through transition to the juvenile stage? 3) What is the evidence for morphological correlates of complex neuromast patterns with microhabitat among *Elacatinus* and *Tigrigobius* species?

MATERIALS AND METHODS

Material Examined

Mated pairs of *E. lori* and *E. colini* were collected near Carrie Bow Caye, Belize (16° 48' 09"N, 88° 04' 55"W) and maintained in a flow-through seawater laboratory at the International Zoological Expeditions field station on South Water Caye, Belize (16° 49' N, 88° 05' W), or in a recirculating seawater system at Boston University, USA. Ontogenetic series of *E. lori* were reared from hatch to settlement (Table 1.1) and were fed zooplankton. A more detailed description of methods can be found in Majoris et al. (2018a).

Additional wild post-settlement *E. lori* and *E. colini* (WC settlers) were collected from reef habitats within the South Water Caye Marine Reserve. Fish were immersed in cold seawater (2–4°C) for 2 min and then fixed in cold (2–4°C) 10% formalin in seawater (or in phosphate-buffered saline; PBS) for subsequent anatomical study, which is consistent with American Veterinary Medical Association guidelines on euthanasia of small warm-water fish (BU IACUC protocol# 16-001). Care was taken to ensure that fish did not contact ice directly. Field research in Belize and the export of samples from Belize was carried out with the approval of the Belize Fisheries Department.

Additional museum specimens of nine species of *Elacatinus* and *Tigriobius* from different microhabitats were obtained for comparative analysis using SEM (coral dwellers - *E. randalli* (n=1), *T. dilepis* (n=1), *T. inornatus* (n=2), *T. pallens* (n=1), and *T.*

zebrella (n=2); sponge dwellers - *E. horsti* (n=1); other microhabitats - *E. punctulatus* (n=1), *T. gemmatus* (n=2), and *T. multifasciatus* (n=2); Table 1.1).

Vital Fluorescent Staining of Neuromasts

A total of 22 live larval *Elacatinus lori* (0 [n=4], 10 [n=4], 20 [n=5], 31 [n=5], and 38 [n=5] days post-hatch [dph]; 3 mm notochordal length [NL] to 9.5 mm standard length [SL]) and adult specimens (n=4, wild-caught, 42-62 mm SL) were stained with 4-di-2-ASP to visualize the number and distribution of SNs under epifluorescence (Figure 1.4; Nakae et al., 2012). Fish were placed in 0.0024% 4-di-2-ASP (4-(4-(diethylamino)styryl)-N-methylpyridinium iodide; Sigma Aldrich) in tank water for 5 minutes. They were then anaesthetized in 0.02% buffered MS-222 (Tricaine methanesulfonate; Sigma Aldrich), and pinned in a Petri dish lined with silicone and filled with MS-222 solution. Specimen were imaged using a Nikon SMZ 1500 dissecting scope equipped with epifluorescence (GFP filter set), a Spot digital camera (Model 25.22, Mp Color Mosaic), and Spot software (v.5.0; Diagnostic Instruments, Sterling Heights, MI).

Neuromast distribution maps were created using fluorescent images of the neuromasts on the head, trunk and tail (in lateral, dorsal and ventral views; Fig 1.4). Maps were annotated with SN line terminology from Sanzo (1911) and Wongrat and Miller (1991). The number of neuromasts per line and the total number of neuromasts were recorded for each individual. Some individual variation within lines of SNs in fishes of the same age (as observed by Marshall, 1972) was observed. Therefore, composite maps were created using mean number of neuromasts in each line for all individuals of the same age.

Histology

Histological material was used to visualize the cranial lateral line canals, canal pores, neuromasts [CNs and SNs], and to confirm the neuromast locations revealed in fluorescence images (above, Figure 1.5). In *E. lori*, the histological material was previously prepared (Hu et al., 2018) and included an ontogenetic series (n=17, 0-44 dph, 3 mm NL - 11 mm SL; 2016) and a wild-caught settler (n=1, 14 mm SL, 2015). Fish > 6 mm SL were decalcified in Cal-Ex (Thermo Fisher Scientific; www.thermofisher.com) for 2 h (6–7.5 mm SL), 3.5 h (8–8.5 mm SL), or 7–8 h (> 8.5 mm SL) and rinsed in PBS for 2 h. All fish were then dehydrated in an ascending ethanol series (to 95% ethanol), infiltrated overnight in glycol methacrylate resin (Technovit 7,100, Electron Microscopy Sciences; www.emsdiasum.com). Individual fish were first embedded in small resin blocks and then 6–8 small resin blocks were re-embedded in a single larger block of resin to allow sectioning in the transverse plane (cross-sections). Sections (5 µm thickness) were cut on a Leica 4M2265 motorized microtome with tungsten carbide knife and individually mounted out of distilled H₂O onto clean slides. The slides were air-dried overnight and stained with 0.5% aqueous cresyl violet for 5 min, rinsed in running tap water, air-dried overnight and coverslipped with Entellan (Electron Microscopy Sciences).

Two wild-caught, post-settlement *E. lori* (14 and 17 mm SL; 2011) were prepared for paraffin histology. These fish were decalcified in Cal-Ex for 7–8 h and rinsed in PBS for 2 h. Then they were dehydrated in ascending series of ethanol and t-butyl alcohol, infiltrated in two changes of Paraplast (Thermo Fisher Scientific) for 4 h under vacuum and individually embedded. Blocks were sectioned at a thickness of 8 µm, mounted on

slides subbed with 10% albumin in 0.9% NaCl, stained with a modified Hall-Brunt quadruple (HBQ) stain (Hall, 1986), and coverslipped with Entellan.

All data were taken from the right side of all fish (assuming symmetry), but data from the left side were used if the right side was damaged [EL153, 44 dph]. In order to document the location of neuromasts, an outline of a fish was drawn to scale (in dorsal, lateral, and ventral views). Each neuromast was drawn on the distribution map as it was observed, assigned a number and the initial and final serial sections in which the neuromast was visible were recorded to determine its rostral-caudal dimension (“length”). The ends of a neuromast were identified by the thickening of the epithelium relative to the general squamous epithelium. The rostral-caudal length of each neuromast was calculated by multiplying total number of serial sections in which a neuromast was present by section thickness (5 or 8 μm , plastic or paraffin respectively).

The maps of neuromasts generated using histological material was compared to the fluorescence maps (see above) as well as published images of neuromast distributions from related taxa (Sanzo, 1911; Marshall, 1972; Hoese & Reader, 2001). The lines and series of SNs and CNs were then annotated using the terminology from Sanzo (1911) and Wongrat & Miller (1991). Within a line, neuromasts were numbered from dorsal to ventral (vertical lines) or rostral to caudal (horizontal lines).

Scanning Electron Microscopy

Scanning electron microscopy (SEM) was used to reveal neuromast shape, size, hair cell orientation and to confirm neuromast locations found in fluorescence and

histology (for *E. lori* specimen). An ontogenetic series of lab reared *E. lori* (n=32, 0-45 dph, 2.5 mm NL to 11 mm SL) and wild caught post-settlement adults of *E. lori* (n=5, 9-50 mm SL) and *E. colini* (n=5, 9-13.5 mm SL) had been fixed in 10% formalin in 0.1 M PBS. Museum specimens in *Elacatinus* and *Tigrigobius* (AMNH specimens n=13, 12 – 24 mm SL, see Table 1.1) also had been fixed in formalin and transferred to 70% ethanol for storage.

All specimens were dehydrated in an ascending ethanol series at room temperature, critical point dried out in liquid CO₂ (Tousimis Sam-Dri 780A), mounted on aluminum stubs with adhesive carbon discs, and coated with platinum (15 nm; Leica MED 020). Specimens were viewed with a Zeiss NTS Supra 40VP SEM at 3 KV and a working distance of ~10 mm. SEM images were used to measure neuromast length, width, area and sensory strip area using ImageJ (Schindelin et al., 2012) and to determine neuromast number and distribution for previously unexamined *Elacatinus* and *Tigrigobius* species. The SN lines were labeled following Sanzo (1911). Museum specimens were in variable condition and did not yield complete neuromast distribution, but did provide some data for analysis.

MicroCT Imaging

MicroCT (μ CT) imaging was used to confirm canal presence/absence observed in histology and the morphology of bony canals (Figure 1.6). Specimens were imaged at the Museum of Comparative Zoology at Harvard (Bruker SkyScan 1173). Canal

presence, absence, and degree of ossification were also confirmed by examining 2D cross sections and μ CT data reconstructed using OsiriX (v3.6.1 64 bit, volume rendering).

Statistical Analysis

Neuromast counts obtained from both epifluorescence and histology were compared using a t-test to determine if the two methods produced comparable results. The sizes (length, width, and area) of neuromasts in post-settlement *E. lori* measured from SEM images were compared using a series of t-tests ($\alpha \leq 0.05$) to determine if there were significant differences between neuromast types (Head SNs, Trunk SNs, Tail SNs, CNs, and CN homologs). The data on SN lines in *Elacatinus* and *Tigrigobius* derived from SEM images and the literature (Miller, 1972; Hoese & Reader, 2001) were compiled for all species examined. SN lines with more than four missing values (e.g., counts not attainable via SEM) were excluded from further analysis. Sixteen of the twenty-five SN lines observed on the cheek in lateral view (Table 1.2, see Figure 1.7 for SN line locations), which had less than 5% of SN count values as unknowns (missing values due to tissue damage, SEM mounting angle, etc.) were used for this analysis. Multidimensional scaling (MDS) and Analysis of Similarity (ANOSIM), non-parametric tests, were used to test for significant differences between genus, SN count, and microhabitat (“sponge”, “coral,” or “other”). These data were input as “abundance” with species data as “rows” and unknown values as “missing” and habitat was added as a factor (using PRIMER v.6 software). The missing number algorithm function was used to generate values for the few remaining unknown SN counts using predictions based on known values for the species and interspecies comparisons (Table 1.2). An MDS plot was

created using Euclidean distance, which showed distinct clustering of sponge and coral species based on SN counts (Figure 1.8). An ANOSIM test was performed using microhabitat type (sponge vs. coral vs. other microhabitat) and genus (*Elacatinus* vs. *Tigrigobius*) to determine if the clustering pattern revealed in the MDS plot was an artifact of taxonomy (genus) or if it was due to microhabitat differences. A Similarity Percentage test was then done for each SN line to determine which specific SN lines explained most of the variation observed in SN number in species occupying different microhabitats.

RESULTS

LL Morphology of Adult *E. lori*

The cranial lateral line system of *Elacatinus lori* is characterized by a reduction of canals (Webb, 2014b) and a proliferation of SNs, which is typical of most gobies (Figure 1.7). *Elacatinus lori* has a transverse SN pattern (SN lines under the eye radiate away from the orbit; Miller et al., 1980), which is similar to other *Elacatinus* and *Tigrigobius* previously described (Miller, 1972; Hoese & Reader, 2001). A total of eight CNs were found in the cranial canals (on one side of the head) in the three adults examined using histology. Fluorescent imaging revealed 318 SNs (± 12 SNs) in four adult fish. SEM showed that all neuromasts in adult *E. lori* (SNs and CN homologs) have an elongate diamond shape with an oval sensory strip in the center; its long axis is perpendicular to the long axis of the neuromast. The axis of best physiological sensitivity of the hair cells in each neuromast (as determined by kinocilium placement relative to the stereocilia in each hair cell) is also perpendicular to the long axis of the neuromast (Figure 1.9) in all neuromasts. Some variation was observed in the shape of the diamond-shaped neuromasts (e.g., robust or more slender diamond-shaped neuromasts).

SEM images revealed that the mean length of SNs (long axis) on the head in post-settlement *E. lori* was 42.28 ± 20.22 μm ($n=102$) and the mean dorsal-ventral width (short axis) was 20.24 ± 11.67 μm ($n=102$). Similarly, the mean length of cranial CNs in post-settlement *E. lori* was 32.26 ± 7.50 μm ($n=13$) but the mean dorsal-ventral width was 21.25 ± 6.92 μm ($n=14$). In comparison, the mean length of cranial CN homologs in post-settlement *E. lori* was 42.43 ± 22.64 μm ($n=45$), but the mean width was 23.76 ± 17.28 μm

(n=45). Thus, the SNs and CN homologs are not significantly different in length, but neuromasts in both SNs and CN homologs are significantly longer than the CNs ($p=0.001$ and $p=0.014$, respectively). The CNs and CN homologs tended to be wider than the SNs, but did not show a statistically significant difference (Table 1.3). Canal neuromasts are the shortest neuromasts and while the CN homologs and SNs are the longest neuromasts on the head. Trunk SNs are significantly narrower than all other neuromast types except the tail SNs. Canal neuromast homologs are the largest neuromasts with respect to area while trunk SNs and CNs are the smallest neuromasts (Table 1.3 and 1.4).

The Lateral Line Canals and Canal Neuromasts

The three cranial canals that are commonly present in gobies (supraorbital, postotic, and a portion of the preopercular canal) are well-developed and ossified in histological specimens of *E. lori*. The mandibular canal (or mandibular portion of the mandibular-preopercular canal) is absent (Figure 1.6). Histology shows that five CNs are located in the supraorbital canal (located in the nasal and frontal bones), two CNs are in the preopercular canal (in the preopercular bone), and one CN is in the postotic canal (located in the post-temporal bone). In the largest specimen examined histologically (wild-caught post-settlement, 14 mm SL), the supraorbital, preopercular and postotic canals were enclosed (Figure 1.5), but did not appear to be fully ossified (Figure 1.6). Ten canal pores are present on the head, and two of those pores (λ and κ , named as per Sanzo, 1911) are common pores that represent the point where the left and right supraorbital canals meet between the orbits. The pores in the supraorbital and postotic canals (in rostral to caudal order) are the σ , λ , κ , ω , α , β , and ϱ pores as per Sanzo (1911).

The three pores in the vertical portion of the preopercular canal are the δ , Y, and ϵ pores (as per Sanzo, 1911; Figure 1.7). Interestingly, two preopercular canal pores were not easily visible in SEM, but were clearly visible in all post-settlement individuals examined histologically (EL 14, EL 17, and EL 041), as well as in one cleared and stained specimen (wild caught, G6 male adult), and in one specimen examined using μ CT (Figure 1.6).

Superficial Neuromast Distribution in Adult *E. lori*

Between 128 and 155 SNs were observed on one side of the head (dorsal, lateral, and ventral) in adult *E. lori* (n=4, 42-62 mm SL) using fluorescence imaging and between 124 and 150 SNs were identified in histological material (n=3, 14-17 mm SL). These two methods did not produce significantly different counts (T-test, p=0.134). Thus, all data reported below are from fluorescence imaging data unless otherwise stated.

Superficial neuromasts were densely placed within lines on the head, trunk, and tail in *E. lori*. Of the nine series identified by Sanzo (1911) on the head, trunk and tail of gobies, eight of them are present in *E. lori* (Figure 1.7 and 1.10):

(1) The Preorbital series (on the “snout”, rostral to the eye) is composed of line “r” (0-3 SNs), line “s₁” (1-3 SNs), line “s₂” (1-3 SNs), and line “c²” (3-4 SNs).

(2) The Suborbital series (running below the eye, on the cheek) in *E. lori* is transverse and is composed of line “1” (1 SN), line “2” (2-3 SNs), line “3” (3-4 SNs), line “4” (2-4 SNs), line “5” (7-9 SNs), line “6” (2-3 SNs), line “7” (1 SN), line “b” (2-5 SNs), and line “d” (12-14 SNs).

(3) The Preoperculo-mandibular series (running along the ventral surface of the lower jaw and on the horizontal portion of the preopercular bone) is composed of line “e” (14-26 SNs), line “f” (2-3 SNs), and line “i” (19-24 SNs). Line “i” is rostral to the first pore of the preopercular canal, which is located in the preopercular bone.

(4) The Opercular series (on the opercular bone) is composed of line “oi” (3-6 SNs), line “os” (4-6 SNs), and line “ot” (12-13 SNs).

(5) The Oculoscapular series (extending from a point caudal to the orbit to the dorsal margin of the base of the pectoral fin) is composed of line “x1” (1-4 SNs), line “x2” (4 SNs), line “u” (4-6 SNs), line “z” (2-4 SNs), line “as1” (3 SNs), line “as2” (3-5 SNs), line “as3” (3-4 SNs), line “la1” (1-2 SNs), and line “la2” (0-2 SNs). In this study, we classified neuromasts rostral to the edge of the operculum to be on the head and all neuromasts caudal to the edge of the operculum to be on the trunk. In this series, lines “as1-3” and “la1-2” were considered trunk SN lines while all the rest were head SN lines.

(6) The Anterior Dorsal series (extending caudally from the eye to the anterior insertion of the dorsal fin) is composed of line “g” (2-3 SNs), line “h” (1-5 SNs), line “n” (3-10 SNs), and line “o” (3-4 SNs).

(7) The Interorbital series (region between eyes) is not present in *E. lori*.

(8) The Body series (on the trunk) is composed of dorsal, median, and ventral subsets of lines (Figure 1.10). There are three short dorsal lines extending dorso-ventrally, located just ventral to the first dorsal fin: line “ld” (5-6 SNs), line “ld1” (2-3 SNs), and line “ld2” (2-3 SNs). *Elacatinus lori* does not have an enclosed trunk canal, but short median SNs lines (“stitches”) sitting over each myomere are present where a lateral

line scale containing a canal would typically be found in other fishes. There are 19-22 short median lines, vertical rows of three to five SNs, along the mid-flank of the fish at the horizontal septum, comprising a total of 65-80 SNs. Lastly, there are five or six short ventral lines extending dorso-ventrally, which are located caudal to the pectoral fin on the lower third of the body: line “lv” (6-14 SNs - potentially obscured by pectoral fin), line “lv1” (2-5 SNs), line “lv2” (5-8 SNs), line “lv3” (5-6 SNs), line “lv4” (5-8 SNs), and line “lv5” (0-2 SNs). The anterior portion of the trunk (behind the pectoral fin) has all three groups of body lines (dorsal, lateral, and ventral), while posterior to the pectoral fin is primarily just the highly organized stitches of the lateral series on each myomere.

(9) The Caudal series (on the caudal fin) is composed of three lines that extend rostro-caudally and sit on the membranes between caudal fin rays: line “lc” (9-16 SNs), line “lc¹” (6-16 SNs), and line “lc²” (12-26 SNs). At the base of the caudal fin rays, there is a short dorsal-ventral line of four SNs arranged tip-to-tip (see below). The bottom two SNs are closer together and both are a part of line “lc²”. The median line, line “lc¹”, is in the second adjacent fin ray membrane to line “lc²” and the dorsal most line, line “lc”, is in the third adjacent fin ray membrane from line “lc¹” (Figure 1.4G). Radiating from these first few caudal SNs are three long lines of relatively equally spaced SNs, about 100 µm apart, aligned side-to-side that extend to the end of the caudal fin (Figure 1.9). When observing the caudal fin using epifluorescence, SNs on both sides of the fin could be seen because the fin membrane is so thin, so SN counts may have been overestimated (Figure 1.4).

Arrangements of SNs Within Lines

All neuromasts (CNs and SNs) are of the same diamond-shape; hair cell orientation (axis of best physiological sensitivity) is parallel to the long axis of the oval sensory strip (comprised of hair cells), which is perpendicular to the long axis of the neuromast. However, the neuromasts in *E. lori* have two different arrangements within lines on the head, trunk and tail (Figure 1.11). Superficial neuromasts on the head and trunk are always aligned “tip-to-tip” with the *long axis* of the neuromast parallel with the line of neuromasts. The long axis of the oval sensory strip and hair cell orientation are perpendicular to the long axis of each neuromast (Figure 1.9 and 1.11A). In contrast, the SNs on the caudal fin are aligned “side-by-side”, with the *short axis* of the neuromast parallel to the line in which the SNs are found (Figure 1.11B). The CNs within canals and hypothesized CN homologs on the skin are also arranged “side-by-side” so that the *short axis* of the neuromast (and thus the axis of best physiological sensitivity of the hair cells) is parallel to the axis of the canal (Figure 1.11C).

Ontogeny of SN Proliferation in *E. lori*

The pattern of neuromast proliferation in *Elacatinus lori* appears to be simple (Figure 1.12). Adult *E. lori* have 275-304 neuromasts (CNs and SNs), but at hatch (0 dph) only 22 neuromasts are present, found in only six of the eight SN series, each with only a few of the total SN lines found in adults. Only 11 neuromasts are present on the head, including two supraorbital and one preopercular presumptive CN and eight SNs. Of the 33 SN lines on the head in adults, only eight are present at hatch, with one SN in each of the eight lines (lines “r”, “c”, “5”, “b”, “i”, “u”, “n”, and “h”). At hatch, many of the neuromasts are not round (like neuromasts in non-gobies; zebrafish - Webb & Shirey,

2003), but are already diamond-shaped and continue to elongate and increase in size throughout ontogeny (Figure 1.13). On the head, the number of SNs increases linearly ($R^2=0.98$) through ontogeny (Figure 1.14). The number of cranial CNs shows a logarithmic trend ($R^2=0.74$); all eight presumptive CNs appear to be present by 15 dph (~5 mm SL; Figure 1.14). The number of lines and the number of SNs within each line on the head and trunk increases with age (Figure 1.12). All 33 SN lines are not present until settlement (30-40 dph, ~7-10 mm SL); the SN lines in the Oculoscapular series are the last to appear in epifluorescence. On the caudal fin, the SN number begins to increase within each of the three lines at 20 dph (in post-flexion larvae, Figure 1.12).

Comparative morphology of the LL System in *Elacatinus* spp. and *Tigriobius* spp.

Five species of *Elacatinus* and six species of *Tigriobius* were examined using SEM to determine neuromast shape, number, and distribution. *Elacatinus* and *Tigriobius* have a longitudinal SN configuration (Miller et al., 1980) with 7 SN lines radiating from the lower margin of the orbit. Overall, the pattern and distribution of the neuromasts was similar; all eight series of SNs found in *E. lori* are present in all other species. The double line of the Preopercular-Mandibular series (lines “e” and “i”) and the Opercular series (lines “ot”, “oi”, and “os”), which form an “F” configuration on the cheek (Figure 1.15), were visualized in all specimens in which the cheek was in good condition (e.g., data not available for *E. horsti* and *T. zebrella*). All species examined appeared to have SNs on papillae.

Neuromast morphology in all *Elacatinus* and *Tigrigobius* species examined was similar to that in *E. lori*. They have elongate diamond-shaped neuromasts and an oval central sensory strip with its long axis perpendicular to the long axis of the neuromast (Figure 1.15). Hair cell orientation was successfully determined only in those museum specimens that were in good condition. For instance, SNs were aligned “tip-to-tip” with the long axis of the neuromast parallel to the line in which the neuromast sits, with the axis of best physiological sensitivity of hair cells perpendicular to the neuromast line. As in *E. lori*, those neuromasts hypothesized to be CN homologs were aligned “side-to-side” with the axis of best physiological sensitivity of hair cells parallel to the neuromast line and to the ancestral canal axis.

Partial SN counts were obtained from SEM images for most of the museum specimens examined (Table 1.2), but damage and skin shrinkage due to overall specimen condition and SEM preparation artifact, limited the ability to measure the length of the sensory papilla (height of SN above skin surface). SEM analysis revealed that all species examined had a supraorbital canal with at least four pores and a preopercular canal with at least two pores. It is likely that all species had five supraorbital canal pores, but not all could be visualized in *T. zebrella* and *T. inornatus* due to specimen condition and orientation of the images obtained.

The comparison of SN counts in a subset of lines (see Methods) with microhabitat yielded results indicating that microhabitat (“sponge”, “coral”, or “other”) had a significant effect on lateral line morphology ($p=0.026$), while genus (*Elacatinus* vs. *Tigrigobius*) did not ($p=0.25$) (Figure 1.8). More specifically, “sponge” dwelling species have significantly more SNs in the lines compared than do “coral” dwelling species

($p=0.004$) but there were no significant differences between species from “sponge” and “other” nor “coral” and “other” microhabitats ($p=0.532$ and $p=0.381$, respectively). This result was likely not due to confounding based on genus because the differences based on genus were low ($p=0.25$). The greatest differences in lateral line morphology between species occupying “sponge” and “coral” microhabitats are attributed to differences within the lines “ot”, “5”, “c²”, “x2”, and “os”, which represent 71.22% of the differences observed between “sponge” and “coral” groups.

DISCUSSION

This study described the mechanosensory lateral line system of *Elacatinus lori*, a coral reef fish with a pelagic larval stage. It is the first to fully describe the ontogeny of the lateral line system in a goby. Even though a few studies have examined the structure of SNs in gobies using SEM (Marshall, 1986; Rouse & Pickles, 1991; Bassett et al., 2006; and Watanabe et al., 2010), this was first study to examine and distinguish between SNs and CNs in larvae and juveniles and to document neuromast arrangement within lines with reference to neuromast shape and hair cell orientation.

This study showed that at hatch, *Elacatinus lori* has 22 neuromasts distributed on the head and body, which is comparable to that in other fishes (Blaxter et al., 1983; Webb & Shirey, 2003; Becker et al., 2016). However, instead of having round neuromasts (like those of the early larvae of other species; zebrafish, Webb & Shirey, 2003; barramundi, Mukai et al., 2007; cichlids, Becker et al., 2016; brown-marbled grouper, Mukai & Seng Lim, 2016), at hatch most of the cranial, trunk and tail neuromasts have already begun to acquire the diamond-shape characteristic of all of the neuromasts in adult *E. lori*. The early appearance of this modified neuromast morphology may have interesting implications for lateral line function in the pelagic larvae of *E. lori*. This study also showed that SNs and CNs are similar in their size and shape, which is relatively unusual (trout, Jones & Webb, unpub. data; cichlids, Becker et al., 2016).

In *E. lori*, all SNs have the same diamond-shape morphology, but within lines, neuromasts show two different arrangements. Superficial neuromasts are always aligned

tip-to-tip while CNs in larvae, presumptive CNs not yet enclosed in canals, are aligned side-to-side (see Fig. 1.9 and 1.11). This indicates that SN lines are sensitive to water flows perpendicular to the axis of the neuromast line and that CNs are sensitive to water flows parallel to the neuromast line (along the length of the canal). This distinction was observed in double line of SNs on the mandible in the current study (see Fig. 1.9) and in other goby taxa (Marshall, 1986; Bassett et al., 2006). The more lateral mandibular line (line “e”, see Fig. 1.7) was typical of other SN lines on the head with neuromasts aligned tip-to-tip and the axis of best physiological sensitivity of all neuromasts perpendicular to the line. In contrast, the neuromasts in the more medial SN line (line “i”, see Fig. 1.7) were arranged side-to-side with the axis of best physiological sensitivity of all neuromasts parallel to the line. Marshall (1986) suggested that this medial line of SNs in the black goby (*Gobius niger*), was the homolog of a line of CNs in the mandibular canal, which has been lost in most gobies. He predicted that these CN homologs retain the functional sensitivity and arrangement of CNs even though they are no longer in a canal. A similar finding in *E. lori* supports this suggestion. Thus, it is concluded that the SNs in line “e”, whose orientation is tip-to-tip, are likely to be “accessory” SNs to the CN homologs in line “i”, which have an axis of best physiological sensitivity perpendicular to the line in which they sit and to that of the CN homologs. The interpretation of neuromasts in line “i” as CN homologs is also supported in most primitive extant gobiform taxa (*Rhyacichthys aspro*, Asaoka et al., 2014), which retains its mandibular canal and the SNs in line “e”, but lacks line “i”.

A similar arrangement is found in line “u”, which extends rostro-caudally, and sits caudal to the last canal pore in the short postotic canal (pore q). It is concluded that the

neuromasts in this line are also CN homologs that had been enclosed in the portion of the postotic canal in more primitive taxa but that is absent in *E. lori*. This is supported by the innervation pattern that Wongrat and Miller (1991) observed in this region in eleotrids. Furthermore, it had been previously noted that gobies with either longitudinal or transverse SN patterns on the cheek both maintain lines “b” and “d” in the same rostral-caudal line orientation, with SNs arranged tip-to-tip (Wongrat & Miller, 1991). Wongrat and Miller (1991) suggested that this may indicate that these two lines are homologous with ancestral lines of SNs while lines “a” and “c” in species with a transverse SN pattern or “1-7” in species with a longitudinal pattern may be lines of CN homologs that have proliferated.

Neuromasts on the trunk of *E. lori* are arranged in what appears to be two distinct patterns. The anterior portion of the trunk is separated into dorsal, lateral, and ventral lines while the posterior portion of the trunk had very neat stitches in each body segment running along the midline to the caudal peduncle (Figure 1.10). This apparent distinction could potentially be explained by the location of the SN lines relative to the large pectoral fins, which likely cover the SNs on the anterior portion of the trunk, which are potentially stimulated during fin movement.

In contrast to the cranial and trunk SNs that are aligned tip-to-tip, the SNs in the three lines on the caudal fin (tail) in *E. lori* are oriented side-to-side; their long axis is oriented dorso-ventrally, but the axis of best physiological sensitivity of their hair cells is oriented rostral-caudally (Figure 1.9F). Lines of SNs on the tail with axis of best physiological sensitivity in the rostral-caudal axis have been seen in other species (e.g., *Aulonocara* and *Tramitichromis*, Bender & Webb unpubl. data; *Rhinogobius* sp.,

Watanabe et al., 2010). This likely relates to the ability of the SNs to detect flow stimuli created by the movements of the body as well as by the median and caudal fins during swimming. In gobies, this may also be important to the detection of flows during station-holding on the substrate (using their pelvic sucker disks), or in the case of *E. lori*, on the inner surface of their host sponges, which generate water flows as they filter feed.

The ability to image the neuromasts and hair cells of museum specimens using SEM also demonstrates that they are a useful source of data for the analysis of the lateral line system if carefully collected and preserved. A comparison of the lateral line system in sister genera (*Elacatinus* and *Tigrigobius*) showed correlations between lateral line organization and microhabitat. Overall, the presence of the diamond-shape neuromast morphology among goby species is highly conserved given its distribution in the American Seven-spined gobies (*Elacatinus* and *Tigrigobius*, this study; *Gobiosoma ginsburgi*, pers. obs.), Ponto-Caspian and Mediterranean gobies (*Gobius niger*, Marshall, 1986), Crested gobies (*Coryphopterus glaucofraenum*, pers. obs.), and Inshore gobies (*Bathygobius fuscus*, Rouse & Pickles, 1991). This morphology was also observed in other species belonging to the gobiiform families Oxudercidae (*Rhinogobius* sp., Watanabe et al., 2010) and Eleotridae (*Gobiomorphus cotidianus*, Bassett et al., 2006). This indicates that the elongate diamond-shaped neuromasts, and their stereotyped arrangements within lines, may be a feature of a larger phylogenetic group within the Gobiiformes.

Why do these gobiiform fishes have diamond-shaped neuromasts and a dramatic proliferation? There are no biomechanical or physiological studies to date that can explain this and the only study that modelled superficial neuromast function used small

round neuromasts in larval zebrafish (Van Trump & McHenry, 2008; Yoshizawa et al., 2015). The base of the cupula reflects the shape of a neuromast, so the most striking difference between round and elongate diamond-shaped neuromasts is the wing-like extensions of the cupula that extend to the tips of the long axis of the elongate diamond-shaped neuromasts. These “wings” are more similar to a sail or flag than to the cylinder previously used in modeling studies (McHenry, pers. comm.). These cupulae present more surface area to flows moving towards the axis of best physiological sensitivity of the hair cells, which likely increases the sensitivity of the neuromast when compared to the cylindrical cupulae of round neuromasts. Rouse & Pickles (1991) suggested that the concentration of hair cells in the center of the neuromast (in the sensory strip) creates a stiffening effect that would prevent the folding over of the extremely long cupula. This idea is consistent with modeling data that shows an increase in cupular stiffness with the addition of more hair cells (McHenry & van Netten, 2007).

The embryological origin and pattern and mechanism of proliferation of neuromasts is well studied only in zebrafish (*Danio rerio*). It has been shown that neuromasts originate from several epidermal placode-derived primordia that deposit neuromasts in waves during embryonic development (Ghysen & Dambly-Chaudière, 2002). Neuromasts then proliferate via budding during the larval and juvenile stages (Ledent, 2002) to reach their adult numbers and distributions. In contrast to *E. lori*, neuromasts present at hatch in other species are small and round with only a few hair cells (e.g. zebrafish, Webb & Shirey, 2003; barramundi, Mukai et al., 2007; cichlids, Becker et al., 2016; brown-marbled grouper, Mukai & Seng Lim, 2016). The neuromasts may then grow and change shape during development (Webb, 2014a). Becker et al.

(2016) suggested that the timing of ontogenetic changes in neuromast shape may be of functional significance. The presence of diamond-shaped neuromasts at hatch in *E. lori* suggests that those neuromasts are more sensitive than those of the round neuromasts in other species. The rapid proliferation of neuromasts in *E. lori* during the larval stage may have additional implications for survival because an increased number of neuromasts will likely increase overall sensitivity of the lateral line system. Superficial neuromasts are thought to function in rheotaxis (Montgomery et al., 2014) and perhaps having a larger number of SNs during the early larval stage is important for larval orientation. Future studies should include modeling of neuromast of various shapes (e.g., diamond, oval, rhomboid), sizes (e.g., small vs. large), patterning (e.g., clusters, lines, solitary) and arrangements (tip-to-tip, side-to-side), as well as behavioral studies of the response of larvae to localized flow stimuli in order to further understand the role of neuromasts in the behavior of pelagic fish larvae (Majoris et al., in prep.).

The lateral line system is morphologically diverse among fishes and some studies have used adaptation to ecological and environmental conditions to explain this diversity. Wark and Peichel (2010) found distinct differences between benthic and limnetic populations of sticklebacks, with benthic fish having more neuromasts on the trunk than limnetic fish. Fischer et al. (2013) found intraspecific variation in SN number in the guppy, *Poecilia reticulata*, is correlated with predation levels. Another study found that the habitat generalist *Gobiomorphus cotidianus*, an eleotrid, exhibits morphological variation in the lateral line system that is correlated with habitat type (Vanderpham et al., 2016). They found an increased number of canal pores in the oculoscapular region in individuals inhabiting more turbulent environments and suggested that the variation in

total canal pores (degree of development of canals) is more closely associated with habitat type than is the total number of SNs (Vanderpham et al., 2016). In a comparative study of the lateral line morphology in nine species of ecologically and closely-related triplefins (Family Tripterygiidae; *Forsterygion* and its sister genus *Notoclinops*), Wellenreuther et al. (2010) found that the lateral line systems morphologically divergent, with species inhabiting areas of higher wave exposure tending to have a higher number of CNs and a reduction of SNs and that species with the greatest number of SNs tended to inhabit shallow and sheltered waters, habitats which are often characterized by poor visibility. A few studies do present contradictory results to the high flow, few SNs hypothesis. For example, Carton and Montgomery (2004) found that within the family Pinguipedidae, *Cheimarrichthys fosteri* has many SNs despite living in a turbulent environment, while *Parapercis colias*, which prefers low flow habitats, has few SNs. Beckmann et al. (2010) also found no relationship between habitat (flowing streams or still lakes) and the total number of SNs in 12 species of European Cypriniformes. The selection of microhabitats within a certain flow regime (e.g. sheltering in pools or among rocks within a fast-moving stream environment) may limit the effect of the high flow environment on the morphology and sensitivity of the lateral line system (Vanderpham et al., 2016), requiring that the hydrodynamics of microhabitats are assessed in detail.

The gobies examined in the current study are all coral reef species that also tend to inhabit shallow and relatively sheltered waters, but coral reefs are generally characterized by low turbidity waters with good visibility. As a group, gobies tend to be relatively small occupants of benthic habitats (Patzner et al., 2011). Perhaps the reduction of cranial canals, which facilitates more extensive neuromast proliferation in gobies is the

result of a combination of their small size (reduced canals may be associated with small body size), benthic lifestyle as well as selection of low flow microhabitats on coral reefs. An alternative explanation for a proliferation of SNs (especially in gobies) could be that cranial SNs are an adaptation for planktonic prey detection and capture (Montgomery & Milton, 1993). The gobies in this study that inhabit “sponge” or “other” microhabitats (e.g., *E. lori*, *E. horsti*, and *T. multifasciatus*) are plankton feeders and the “coral” dwellers (e.g., *E. oceanops*, *E. randalli*, and *T. dilelis*) are mostly cleaner fishes. The “sponge” dwellers (e.g., *E. lori* and *E. horsti*) tend to have significantly more SNs in the lines compared than “coral” dwellers. It is possible that the planktivorous gobies have more SNs to allow them to better detect planktonic prey, while cleaner gobies rely more on other sensory systems to detect clients and food items on those clients. The species of *Elacatinus* that are cleaners also tend to be smaller than their non-cleaning congeners (Cote, 2000). The lower number of SNs in coral-dwelling species may be a factor of fish size in that they have a smaller surface area for proliferation of SNs.

In conclusion, the lateral line system of gobies is notably complex and impressive, and so attempts to carry out morphological and functional analyses are rather daunting. However, the morphological distinction between CNs, CN homologs, and SNs, the recognition of the stereotyped morphology and arrangements of neuromasts within lines, and description of how neuromast proliferations develop during the larval stage provided by this study has begun to demystify this complexity thus providing a context for further study. The morphological specialization of all neuromasts (represented by their diamond-shape) so early in the larval phase in *E. lori* suggests that neuromast function is important to their pelagic larvae, although the specific behavioral context (e.g., feeding vs. larval

orientation and navigation) will need to be elucidated in behavioral studies (Majoris et al., in prep.) The comparison of neuromast numbers among species in *Elacatinus* and *Tigrigobius* demonstrates the value of museum material to such studies, and contributes to our appreciation of how interspecific variation in lateral line morphology may be related to functional roles in closely related species that differ in microhabitat and feeding strategies.

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TABLES

Table 1.1 *Elacatinus* and *Tigrigobius* Specimens Examined. AMNH or archival (Webb lab) identification numbers are provided and methods used to examine each fish are noted. (FL- fluorescence).

Species	Specimen ID	N	Size (mm SL)	Age (dph)	FL	Histology	SEM
<i>Elacatinus lori</i>			3	0	X		
<i>Elacatinus lori</i>			4.5	10	X		
<i>Elacatinus lori</i>		5	6-7	20	X		
<i>Elacatinus lori</i>		5	7-9	31	X		
<i>Elacatinus lori</i>		5	8-9.5	38	X		
<i>Elacatinus lori</i>	EL G4 F	1	42	WC	X		
<i>Elacatinus lori</i>	EL G12 F	1	44	WC	X		
<i>Elacatinus lori</i>	EL G1 F	1	44	WC	X		
<i>Elacatinus lori</i>	EL G4 M	1	62	WC	X		
<i>Elacatinus lori</i>	EL 101	1	3	0		X	
<i>Elacatinus lori</i>	EL 003	1	3	1		X	
<i>Elacatinus lori</i>	EL 004	1	3.5	2		X	
<i>Elacatinus lori</i>	EL 005	1	4	3		X	
<i>Elacatinus lori</i>	EL 006	1	4	4		X	
<i>Elacatinus lori</i>	EL 007	1	4	5		X	
<i>Elacatinus lori</i>	EL 008	1	4	6		X	
<i>Elacatinus lori</i>	EL 009	1	4.5	7		X	
<i>Elacatinus lori</i>	EL 010	1	4	8		X	
<i>Elacatinus lori</i>	EL 012	1	4	9		X	
<i>Elacatinus lori</i>	EL 015	1	5	10		X	
<i>Elacatinus lori</i>	EL 029	1	6	15		X	
<i>Elacatinus lori</i>	EL 131	1	6	20		X	
<i>Elacatinus lori</i>	EL 141	1	8	24		X	
<i>Elacatinus lori</i>	EL 032	1	9	30		X	
<i>Elacatinus lori</i>	EL 033	1	9.5	34		X	
<i>Elacatinus lori</i>	EL 153	1	11	44		X	
<i>Elacatinus lori</i>	EL 041	1	14	WC		X	
<i>Elacatinus lori</i>	EL 17	1	17	WC		X	
<i>Elacatinus lori</i>	EL 14	1	14	WC		X	
<i>Elacatinus lori</i>	EL 99		2.5	0			X
<i>Elacatinus lori</i>	EL 52		3.5	10			X
<i>Elacatinus lori</i>	EL 99		6.0	20			X
<i>Elacatinus lori</i>	EL 99		8.0	27			X
<i>Elacatinus lori</i>	EL 99		8.0	30			X
<i>Elacatinus lori</i>	EL 99		9.5	34			X
<i>Elacatinus lori</i>	EL 99		11.0	40			X

<i>Elacatinus lori</i>	EL 99		11.0	45			X
<i>Elacatinus lori</i>		3	9.0-9.5	WC settler			X
<i>Elacatinus lori</i>	EL G10 F	1	38	WC adult			X
<i>Elacatinus lori</i>	EL G10 M	1	50	WC adult			X
<i>Elacatinus lori</i>	EL 105	1	3	2			X
<i>Elacatinus lori</i>	EL 110	3	3-4	4			X
<i>Elacatinus lori</i>	EL 114	2	3.4-4	6			X
<i>Elacatinus lori</i>	EL 118	1	3.5-4	8			X
<i>Elacatinus lori</i>	EL 122	2	4-5	10			X
<i>Elacatinus lori</i>	EL 126	4	4-4.5	14			X
<i>Elacatinus lori</i>	EL 128	2	5	16			X
<i>Elacatinus lori</i>	EL 130	2	4.5-5	18			X
<i>Elacatinus lori</i>	EL 134	1	6	20			X
<i>Elacatinus lori</i>	EL 138	1	5	22			X
<i>Elacatinus lori</i>	EL 140	1	6	24			X
<i>Elacatinus lori</i>	EL 145	2	5.5	28			X
<i>Elacatinus lori</i>	EL 149	1	7	30			X
<i>Elacatinus lori</i>	EL 152	2	7-8	44			X
<i>Elacatinus colini</i>	EC 6	1	9	60			X
<i>Elacatinus colini</i>	EC 6	1	10	60			X
<i>Elacatinus colini</i>	EC 6	1	10.5	70			X
<i>Elacatinus colini</i>	EC 6	1	12	70			X
<i>Elacatinus colini</i>	EC 6	1	13.5	70			X
<i>Elacatinus horsti</i>	AMNH 264763	1	24	WC			X
<i>Elacatinus puncticulatus</i>	AMNH 73449	1	17	WC			X
<i>Elacatinus randalli</i>	AMNH 238786	1	18	WC			X
<i>Tigrigobius dilepis</i>	AMNH 250269	1	14	WC			X
<i>Tigrigobius gemmatus</i>	AMNH 26076	2	15-18	WC			X
<i>Tigrigobius inornatus</i>	AMNH 233779	1	19	WC			X
<i>Tigrigobius inornatus</i>	AMNH 256755	1	22	WC			X
<i>Tigrigobius multifasciatus</i>	AMNH 23621	2	19-22	WC			X
<i>Tigrigobius pallens</i>	AMNH 26071	1	12	WC			X
<i>Tigrigobius zebrella</i>	AMNH 248920	2	18-19	WC			X

Table 1.2 *Elacatinus* and *Tigriobius* SN counts for a subset of cranial SN lines (see Figure 1.7 for locations) using species from different microhabitats (sponge, coral or other) based on SEM. *Elacatinus oceanops* and *T. macrodon* counts are from published lateral line maps (Miller, 1972). Counts in bold were calculated by the missing value command in PRIMER v.6.

Species	s1	r	c2	2	3	4	5	6	7	b	ot	oi	os	x2	u
<i>E. colini</i>	2	3	4	1	2	2	5	1	1	3	12	3	5	5	4
<i>E. horsti</i>	2	2	4	2	3	4	10	2	1	4	9	4.10	3	4	5
<i>E. lori 1</i>	3	2	4	2	3	4	6	4.98	1	4	14	4	4	4	6
<i>E. lori 2</i>	1	0	3	3	3	4	9	3	1	4	12	6	6	4	6
<i>E. lori 3</i>	2	3	3	3	3	2	7	4	1	2	12	4	4	4	6
<i>E. lori 4</i>	2	3	4	2	3	3	9	3	1	4	12	4	4	4	4
<i>E. lori 5</i>	3	3.57	4	3	4	4	9	3	1	5	13	3	4	4	6
<i>E. lori 6</i>	2	3	4	2	2	3	8	4	1	5	11	4	4	5	5
<i>E. lori 7</i>	1	2	4	3	3	4	9	3	1	4	14	5	7	5	6
<i>E. oceanops</i>	2	2	1	3	4	5	9	4	1	5	9	3	3	5	5
<i>E. punctulatus</i>	2	3	4	2	3	4	9	3	1	4	13	3	4	3	4
<i>E. randalli</i>	3	2.44	4	2	3	3	6	3	1	3	7	2	3	0.89	5.48
<i>T. dilepis</i>	2	2	4	2	2.62	3.07	6	3	1	2	8	3	3	3	4
<i>T. gemmatus</i>	2	2	3	2	3	4	9	4	1	5	12	3	4	3	3
<i>T. inornatus</i>	2	3	4	2	3	3	6	3	1	3	10.42	4.12	5.20	5	6
<i>T. limbaughi</i>	2.16	1.79	1	3	3	2	6	3	1	4	12	3	6	2	7
<i>T. macrodon</i>	2.00	2.29	0	2	2	4	5	3	2	5	12	4	6	4	4
<i>T. multifasciatus</i>	2	3	4	2	3	3	8	2	1	3	11	2	4	2	3

Table 1.3. Size and sample size of SNs, CNs and CN homologs (CNH) on the head, trunk and tail in *E. lori*.

NM Type	n	Mean Length + SD (μm)	Mean Width + SD (μm)	Mean Area + SD (μm^2)
Head SN	102	42.28 ± 20.22	20.24 ± 11.67	533.73 ± 553.35
Trunk SN	65	36.88 ± 18.14	15.77 ± 7.78	349.61 ± 343.86
Tail SN	8	42.52 ± 10.55	17.71 ± 5.44	397.65 ± 196.02
Head CN	13	32.36 ± 7.50	21.35 ± 6.92	349.08 ± 146.45
Head CNH	45	42.43 ± 22.64	23.76 ± 17.28	675.02 ± 805.43

Table 1.4. Comparison of lengths, widths and areas among neuromast groups (See Table 1.3) in *E. lori*. T-test p-values are recorded for each comparison between neuromast groups. Significance at $p \leq 0.05$ is indicated by an asterisk. SN = superficial neuromast, CN = canal neuromast, CNH = canal neuromast homolog.

LENGTH					
	Head SN	Trunk SN	Tail SN	CN	CNH
Head SN					
Trunk SN	0.07				
Tail SN	0.956	0.22			
Head CN	0.001 *	0.15	0.04 *		
Head CNH	0.97	0.17	0.99	0.014 *	
WIDTH					
Head SN					
Trunk SN	0.0017 *				
Tail SN	0.28	0.316			
Head CN	0.625	0.013 *	0.2		
Head CNH	0.217	0.004 *	0.068	0.46	
AREA					
Head SN					
Trunk SN	0.009 *				
Tail SN	0.14	0.57			
Head CN	0.0086 *	0.99	0.56		
Head CNH	0.29	0.013 *	0.051	0.013 *	

FIGURES

Figure 1.1 Basic anatomy of the lateral line and neuromasts. **A)** Diagrammatic representation of a neuromast, which is the sensory organ that compose the lateral line system (from Roberts & Ryan, 1971). Neuromasts are composed of a central bundle of innervated sensory hair cells surrounded by non-sensory support cells (sustentacular cells). The hair cells have bundles of stereocilia and a single kinocilia that project into the gelatinous cupula, which covers the entire neuromast. **B)** SEM of a neuromast in the goby *Elacatinus lori* illustrating the central sensory hair cells (sensory strip) in a diamond shaped neuromast. Note that the sensory strip is perpendicular to the long axis of the neuromast and the axis of best physiological activity of the hair cells is parallel to the long axis of the sensory strip (double-headed arrow). **C)** Diagrammatic representation of superficial and canal neuromasts in *Hybopsis aestivalis* (from Webb, 2011). Neuromasts are either located in the epidermis (ep) of the skin, above the basement membrane (bm) as a superficial neuromast (sn) or within the dermis (d), enclosed in dermal bone (dm) that forms pored canals as a canal neuromast (cn), n- nerve. **D)** Skull of the convict cichlid, *Amatitlania nigrofasciatus*, illustrating the cranial lateral line canals typical of teleost fishes (from Webb, 2000). The supraorbital canal is in the nasal (na) and frontal bones (fr), the infraorbital canal is in the lacrimal (la) and other infraorbital bones (io), the preopercular canal is in the preoperculum (po), the mandibular canal is in the dentary (de) and anguloarticular (aa), the otic canal is in the pterotic (pt), the supratemporal commissure is in the lateral (le) and medial extracapsular bones (me), and the postotic canal is in the post-temporal bone (pt).

Figure 1.2 Superficial neuromasts and canal pore distribution in gobies. Neuromasts are small black circles and canal pores are larger open circles. **A)** *Thorogobius macrolepis*, with SN line labels originally identified by Sanzo (1911). *Thorogobius macrolepis* has a transverse SN distribution pattern with lines ventral to the eye (lines a, b, c, and d) running rostral-caudal (drawn from Sanzo, 1911). **B)** *Elacatinus oceanops*, illustrating a longitudinal SN distribution pattern with lines ventral to the eye running dorsal-ventral (lines 1-7), appearing to radiate from the orbit. This is the only published information on the lateral line system of the genus *Elacatinus* as it is currently recognized (drawn from Miller, 1972). **C)** *Tigrigobius limbaughi* (= *Elacatinus limbaughi*), illustrating a longitudinal SN distribution pattern similar to *E. oceanops* (drawn from Hoese and Reader, 2001). **D)** *Tigrigobius macrodon*, illustrating a longitudinal SN distribution pattern similar to *E. oceanops* and *T. limbaughi* (drawn from Miller, 1972).

Figure 1.3 Maximum likelihood phylogeny of American Seven-Spined gobies (Figure 2 and 3 from Taylor & Hellberg, 2005) based on mtcyb, rag1 and rho genes. Values above the branch or to the left of the slash are nonparametric ML bootstrap proportions and values below the branch or to the right of the slash are Bayesian posterior probabilities. See footnote¹ for missing Bootstrap and Bayesian values. Study species for this study are in gray boxes with color-coded micro-habitat (see key). Published tree did not include *E. colini* (sponge) or *T. zebrella* (coral). Sponge-dwelling *Elacatinus* spp. examined are found in a clade that is sister to the clade containing coral-dwellers (*E. oceanops* and *E. randalli*). Both are sister to the clade containing the only Pacific *Elacatinus* species (“other”). Coral-dwelling *Tigrigobius* species are distributed among several clades, but species living in the “other” habitats are in a separate clade, sister to the coral-dwellers.

¹ Bootstrap and Bayesian values missing from Figure 1.3 (from Figures 2 and 3 in Taylor & Hellberg, 2005). *Elacatinus evelynae* (b) = 89/100; *E. evelynae* (b) + *E. evelynae* (w) = 100/100; *E. oceanops* (Belize) = 100/100; *E. oceanops* (Florida) = 100/100; *E. illecebrosus* = 100/100; *E. illecebrosus* + *E. prochilos* = 74/99; *E. prochilos* = 95/100; *E. illecebrosus* + *E. prochilos* = 83/100; *E. horsti* (Curacao) = 100/100; *E. horsti* (Curacao) + *E. chancei* = 99/100; *E. horsti* (Curacao) + *E. chancei* + *E. chancei* = 100/100; *E. louisae* = 100/100; *E. louisae* + *E. horsti* (Bahamas) = -/71; *E. lori* = 100/100; *E. horsti* (Jamaica) + *E. horsti* (Navassa) = 98/100; and *E. horsti* (Jamaica) + *E. horsti* (Navassa) + *E. horsti* (Grand Cayman) = 99/100.

Figure 1.4 Fluorescent images of neuromast distributions in *E. lori*. Specimens were vitally stained with 4-di-2-ASP and shown here in lateral view (rostral to the left). **A)** 0 dph (3 mm NL) individual with only nine neuromasts present on the head. At this stage, the fin-fold and yolk sac are present. By 1 dph, the yolk sac is fully absorbed, and by 10 dph, notochordal flexion is occurring. **B)** 38 dph (9.5 mm SL) individual with all SN lines present on the head, but most have not yet begun to proliferate (except the lines on the operculum and mandible). CNs are still clearly visible, indicating they are not yet fully enclosed in a bony canal. Settlement occurs around this time (30-45 dph, 9-11 mm SL). **C)** Wild caught adult (42 mm SL) with fully proliferated SN lines on the head (see also Figures 1.7 and 1.9). **D)** The trunk and tail of a 20 dph (6 mm SL) individual with only the initial SNs, which will proliferate to become short dorsal-ventral series of SNs on the trunk (“stitches” visible in F). A few SNs on the caudal fin are visible in three lines. **E)** Anterior portion of the trunk of an adult *E. lori* (42 mm SL) illustrating the apparent discontinuity of SN lines in this region of the trunk. **F)** Posterior portion of the trunk of an adult *E. lori* (42 mm SL) with well-organized vertical triplets in association with each myomere along the horizontal septum of the trunk. **G)** Caudal fin of adult *E. lori* (42 mm SL) with three lines of SNs (lines “lc”, “lc₁”, and “lc₂”) extending from the fin base to the tip of the caudal fin, each sitting on the membrane between two adjacent fin rays. The caudal fin membrane is so thin that the SNs from both the left (white arrow head) and right (yellow arrow head) side are visible in this image.

Figure 1.5 Supraorbital (SO) canal enclosure and neuromast structure in *E. lori*. Canal enclosure stages are given following Webb and Shirey (2003). Black arrowheads indicate neuromasts. **A)** Canal neuromast SO3 prior to canal enclosure (Stage I) in a 0 dph individual. **B)** Canal neuromast SO3 beginning canal enclosure (Stage IIa) in a 10 dph individual. Note the two cell layers visible in the neuromast, the upper layer is composed of hair cells. **C)** Canal neuromast SO3 with canal walls (arrows) nearly enclosing it (Stage IIb). **D)** Canal neuromast SO3 after it has been fully enclosed in the canal (Stage IV) in wild caught settler (14 mm SL). **E)** Line of four SNs on the snout (line “c2”) below the nostril (ne = nasal epithelium) of a wild caught settler (14 mm SL). Scale bars = 50 μ m.

Figure 1.6 CT 3-D reconstruction of cranial skeleton of adult *E. lori* in dorsal (A), lateral (B), and ventral (C) views. D) A 45-degree dorsal view of the nasal and frontal bones, providing a good view of the supraorbital canal (SO) and pores. E) A 45-degree ventral view of the dentary and anguloarticular bones, illustrating the lack of mandibular canal in the dentary bone in *E. lori*. Red arrowheads in A, B and E indicate canal pore locations with seven pores in the supraorbital and postotic canals and three pores in the preopercular canal.

Figure 1.7 Diagrammatic representation of reduced cranial canals and canal and superficial neuromast distribution in adult *E. lori* based on fluorescent images of an adult female (44 mm SL) in **A**) dorsal, **B**) lateral, and **C**) ventral views. Neuromasts are small black circles. A total of 7 SN series (major groups of SNs defined by Sanzo, 1911) are color coded: red = preorbital, green = suborbital, yellow = preoperculo-mandibular, orange = opercular, blue = oculoscapular, purple = anterior dorsal, and pink = body. The eighth series in *E. lori* is the caudal series, which is not shown here (see Figure. 1.10). SN lines within series (labeled) are named using Sanzo (1911) and Wongrat and Miller (1991). The position of canals and canal pores (open circles) are illustrated based on data from histological analysis and labeled following Sanzo (1911).

Figure 1.8 MDS plot showing the clustering of microhabitat types based on 16 SN line counts in *Elacatinus* and *Tigri gobius* spp. Distances between points are relative similarities of each species which were calculated using the Euclidean distance (see Table 1.2 for values used in calculation), where closer points had more similar SN counts and distant points were more dissimilar. Microhabitats are indicated by distinct colors and shapes: green triangle = “sponge”, blue inverted triangle = “other”, and teal square = “sponge”. Each specimen has a unique identifier, so replicates of the same species are numbered numerically. The 2-D stress value for this plot was 0.14, which means this visualization corresponds reasonably well with the actual multivariate distance between the samples.

Figure 1.9 SEMs of SNs in patterns present in *E. lori* (rostral to left in all images). **A)** Radiating lines of SNs (lines “2”, “3”, “4”, “5”, “b”, and “d”) on the cheek (see Figures 1.4 and 1.7). **B)** Opercular SN series (lines “ot”, “os”, and “oi”) forming the “F” on the cheek, and preopercular canal pores (ϵ , γ). **C)** Post-otic region of the head (just caudal to the post-otic canal pore) illustrating the upper pair of SNs aligned tip-to-tip and the lower line of SNs extending caudally from the canal pore which are aligned side-to-side. **D)** Double line of SNs on the mandible (ventral view) where the upper line has a tip-to-tip neuromast arrangement and the lower line has a side-to-side neuromast arrangement (see Figure 1.11). **E)** Stitch of three SNs on the midline (horizontal septum) of the trunk (see Figure 1.4 and 1.10). **F)** Middle row of SNs on the caudal fin (line “lc1”) with the SNs aligned side-to-side (see Figure 1.11). Double headed arrows indicate axis of best physiological activity of the hair cells within neuromast. Scale bars = 100 μm in A, B, D; 20 μm in F; and 10 μm in C and E.

Figure 1.10 Neuromast distribution on the trunk and tail of *E. lori* settlers based on fluorescent images of a 38 dph (9.5 mm SL) individual. Neuromasts are small black circles. A total of 4 SN series (major groups of SNs defined by Sanzo, 1911) are color-coded: blue = oculoscapular, purple = anterior dorsal, pink = body, and brown = caudal. The other four series in *E. lori* are on the head, which is not shown here (see Figure. 1.7). SN lines within series (labeled) are named as per Sanzo (1911) and Wongrat and Miller (1991). The pectoral fin was not drawn in order to fully visualize all neuromasts.

Figure 1.11 Neuromast arrangements present in *E. lori* and all gobies examined **A)** SNs are aligned tip-to-tip with the axis of best physiological sensitivity perpendicular to the line of SNs (double-headed arrows). SN lines may radiate in any direction. **B)** CNs or CN homologs (CNH) are aligned side-to-side with the axis of best physiological sensitivity (double headed arrows) parallel to the length of the canal (black lines represent canal walls). **C)** Tail (caudal) SNs are arranged side-to-side with the axis of best physiological sensitivity parallel to the line of SNs (along length of the fin rays). SNs are located on the epithelium between two adjacent fin rays (represented by dashed lines).

Figure 1.12 Neuromast distribution maps of an ontogenetic series of *E. lori* derived from fluorescent images. **A)** 0 dph, 3 mm TL; **B)** 10 dph, 4.5 mm SL; **C)** 20 dph, 6.5 mm SL; **D)** 31 dph, 9 mm SL; and **E)** 38 dph, 9 mm SL pre-settlement *E. lori*. Pectoral fins were removed to facilitate visualization of all neuromasts. Pectoral fins and yolk sacs are not drawn. Scale bar = 1 mm.

Figure 1.13 Neuromast development in *E. lori* (rostral to the left). Neuromasts are ~ 10 μm long at hatch, but most are already diamond shaped. By settlement at ~ 35 dph, neuromasts are significantly elongated, ~ 40 μm long and the sensory strip appears to take up a smaller portion of the entire neuromast area. **A)** 0 dph - neuromast on the trunk. **B)** 10 dph - neuromast on the trunk. **C)** 20 dph - neuromast on the cheek. **D)** 34 dph - neuromast on the cheek. **E)** Adult - neuromast on the caudal fin. Scale bars = 2 μm in A, B, and C; 5 μm in D and E. Double-headed arrows indicate axis of best physiological sensitivity of the hair cells in each neuromast.

Figure 1.14 Neuromast number on one side of head in *E. lori* larvae and settlers (0-44 dph and wild caught settler) based on histological material. SNs= blue. CNs = red. SN counts show a linear trend, $R^2 = 0.9819$, while CN counts have a logarithmic trend, $R^2 = 0.7391$.

Figure 1.15 Neuromast morphology in *Elacatinus* and *Tigrigobius* spp. from museum specimens **A)** *Tigrigobius multifasciatus* showing radiating lines on the cheek (lines “3”, “4”, “5”, “b”, and “d”). **B)** *Tigrigobius gemmatus* showing opercular series (lines “ot”, “os”, and “oi”) and preopercular canal pores (ϵ , γ). **C)** Box in A showing tip-to-tip arrangement of SNs in lines “5” and “b”. **D)** *Tigrigobius gemmatus* showing SN line just caudal to pectoral fin on trunk. **E)** *Tigrigobius dilepis* showing lower opercular line (“os”) with hair cell orientation (double-headed arrows) perpendicular to SN line. **F)** Box in C showing the first 2 SNs in line “b” with tip-to-tip arrangement and hair cell orientation (double-headed arrows) perpendicular to the SN line.

CHAPTER 2

INTRODUCTION

The visual environment is radically different between the water column in which pelagic larvae live and the waters in complex coral reef habitats in which post-settlement juvenile fishes live (Marshall et al., 2003; reviewed in Leis et al., 2011). The spectrum of colors present on coral reefs is much wider than that of the primarily blue pelagic ocean. Thus, it is not surprising that ontogenetic shifts in proportions of rod and cone receptor cells in the retina (Shand et al. 1999a; Shand 1993; Pena & Dumas 2007), the peak wavelengths of cone receptors (Shand 1993), and changes in opsin gene expression (Cortesi et al. 2016; Carleton et al. 2016), have all been demonstrated in species of marine and freshwater tropical fishes. Recently, it was shown that opsin expression in the larvae of a damselfish, *Pseudochromis fuscus*, is dominated by genes for short (UV and violet, 300-450 nm) and medium (blue-green, 450-565 nm) wavelength opsins, while adults had an increased number of opsin genes and increased expression of long wavelength (red, 625-740 nm) opsins (Cortesi et al., 2016). Carleton et al. (2016) also found ontogenetic shifts in single-cone sensitivity from UV to violet to blue in cichlid fishes and suggest that these changes may be correlated with changes in prey type consumed by larvae versus juveniles.

The morphology of the eye, including the retina, has been well-studied in fishes (e.g. *Danio rerio*, *Salmo gairdneri*, and *Poecilia reticulata*, Kunz, 2001). The retina is a multi-layered structure composed of neurons and receptor cells (rods and cones), which are responsible for transduction of light cues. The fish retina is composed of seven layers: amacrine cell, bipolar cell, ganglion cell, inner plexiform, outer plexiform, photoreceptor

(rods, cones), and the pigmented epithelium, which sits at the back of the retina. The pigmented epithelium is capable of responding to light and moves between the photoreceptor cells protecting the cells when light intensity is high (Ali & Anctil, 2012). The photoreceptive layer is composed of rod and cone cells, which are specialized cells that respond to certain wavelengths of light. Rod cells tend to be more numerous and are used for vision in low light (scotopic vision). Cone cells are wavelength specific color receptors (photopic vision). The rods and cones are arranged in a mosaic pattern, which is species specific. The outer plexiform is composed of the fibers of visual cells and dendrites of the bipolar cells. The amacrine and bipolar cell layers are composed of second-order neurons that have lateral processes that interconnect neurons in a certain retinal region. The ganglion cell layer is composed of nerve fibers that converge at the optic disc and form the optic nerve. The inner plexiform is a dense, mesh-like network of neuronal fibers that interconnect the ganglion cells and the amacrine and bipolar cells (Ali & Anctil, 2012). All of the layers of the retina are important in the overall light sensing function of the retina.

There is an overall trend for fish eyes to be almost spherical and increase in size proportionally through ontogeny (Kapoor & Hara, 2001) and the retina enlarges primarily as a result of the addition of rods and cones (Johns, 1981). Since fish continue to grow throughout their lifetime, their eyes and retinas also continue to grow, but the growth rate of the eye and retina during adulthood is much lower than earlier in development (Johns, 1981), which is apparent in the relatively small eyes of large fish compared to the eyes of larval fishes.

Vision is often recognized as the primary sensory modality for many fishes and it has been shown to be important for navigation by pelagic marine fish larvae (reviewed in Atema et al., 2016; Leis et al., 2011). Vision in gobies has been investigated primarily in adults of sand gobies (Gobiidae; Ebert & Andrew, 2009; Larmuseau et al., 2009, 2010), but little work has been done on the ontogeny of goby eyes (gobioid; Senoo et al., 1994). It has been shown that the transforming larvae of *Elacatinus lori* preferentially settle on yellow tube sponges (*Aplysina fistularis*) and can distinguish them visually from sponges of other colors (Majoris et al., 2018a). A shift in opsin expression during the settlement process would suggest that sensory tuning plays a role in the transition from a pelagic (larval) to benthic (adult) environment. The goal of this study is to test the hypothesis that both opsin expression and retinal morphology in *E. lori* will change during the larval period demonstrating sensory tuning, which facilitates visually-mediated settlement site selection.

MATERIALS AND METHODS

Material Examined

Mated pairs of *E. lori* and *E. colini* were collected near Carrie Bow Caye, Belize (16° 48' 09"N, 88° 04' 55"W) and maintained in a flow-through seawater laboratory at the International Zoological Expeditions field station on South Water Caye, Belize (16° 49' N, 88° 05' W), or in a recirculating seawater system at Boston University, USA. Ontogenetic series of *E. lori* were reared from hatch to settlement and were fed zooplankton. A more detailed description of methods can be found in Majoris et al. (2018a).

Additional wild caught post-settlement *E. lori* and *E. colini* (WC settlers) were collected from reef habitats within the South Water Caye Marine Reserve. Field research in Belize and the export of samples from Belize was carried out with the approval of the Belize Fisheries Department. Fish were immersed in cold seawater (2–4°C) for 2 min (ensuring that fish did not contact ice directly) and then fixed in cold (2–4°C) 10% formalin in seawater (or in phosphate-buffered saline; PBS) for subsequent anatomical study or fixed in RNAlater for molecular study. This protocol is consistent with American Veterinary Medical Association guidelines on euthanasia of small warm-water fish and was carried out under Boston University IACUC protocol #16-001.

Morphological Analysis

Histological material (previously prepared: Hu et al., 2018; See Nickles in prep.) was used to measure the growth of the eye (lens, cornea, and retina) and the thickness of retinal layers (amacrine cells, bipolar cells, ganglion cells, inner plexiform, outer plexiform, photoreceptive layer, and the pigmented epithelium) throughout the post-embryonic ontogeny of *E. lori* (Figure 2.1). The histological material was composed of an ontogenetic series (0-44 days post hatch [dph], 3 mm NL - 11 mm SL; n=17; 2016) and an additional wild-caught settler (n=1, 14 mm SL, 2015). Fish > 6 mm SL had been decalcified in Cal-Ex (Thermo Fisher Scientific; www.thermofisher.com) for 2 h (6–7.5 mm SL), 3.5 h (8–8.5 mm SL), or 7–8 h (> 8.5 mm SL) and rinsed in PBS for 2 h. All fish were then dehydrated in an ascending ethanol series (to 95% ethanol), infiltrated overnight in glycol methacrylate resin (Technovit 7,100, Electron Microscopy Sciences; www.emsdiasum.com). Individual fish were first embedded in small resin blocks and then 6–8 small resin blocks were re-embedded in a single larger block of resin to allow sectioning in the transverse plane (cross-sections). Sections (5 µm thickness) were cut on a Leica 4M2265 motorized microtome with tungsten carbide knife and individually mounted out of distilled H₂O onto clean slides. The slides were air-dried overnight and stained with 0.5% aqueous cresyl violet for 5 min, rinsed in running tap water, air-dried overnight and coverslipped with Entellan (Electron Microscopy Sciences).

The initial (rostral-most) and final (caudal-most) sections in which the eye was visible was identified by the presence of extraocular muscle tissue associated with each of the eyes. The center of each eye was identified by finding the midpoint between the initial and final sections. In addition, the size of the lens was determined by identifying the initial and final sections in which the left and right lenses were visible. The sections

in which the center of the eye and the center of the lens were found and, if necessary, the mean center point was used to identify sections for quantitative analysis.

Images of individual histological sections were captured a Zeiss AxioImager 1 microscope using AxioCam software using the 10X objective. The sections 10 μ m rostral and 10 μ m caudal to the section at the midpoint of the eye (see above) were imaged so that three images could be analyzed for each eye in each specimen. Retinal layers (ganglion cell, inner plexiform, amacrine cell, bipolar cell, outer plexiform, photoreceptor, and pigmented epithelium) were identified using the Zebrafish Atlas (<http://zfatlas.psu.edu/>) as a reference. The thickness of the cornea, lens, and retinal layers were measured directly behind the lens (in the center of the eye when possible, Figure 2.1) using ImageJ (Schindelin et al., 2012) for each of the three images per specimen. Each measurement was taken three times and the mean of the three measurements was used for further analysis.

Molecular Analysis

Reverse Transcription quantitative polymerase chain reaction (RT-qPCR) was used to determine relative expression of each of the retinal opsin genes throughout ontogeny in *Elacatinus lori*, thus allowing the identification of any ontogenetic shifts in opsin expression that would represent sensory tuning. To complete RT-qPCR, we first needed to extract DNA, determine opsin gene sequences, extract RNA, and then design and run the RT-qPCR.

DNA extraction was a critical first step because in order to perform RT-qPCR, species specific gene sequences must be identified. In the absence of published opsin sequences for *E. lori* (except the rod opsin, Taylor & Hellberg, 2005), we used opsin sequences of the closest related goby species available (*Amblygobius phalaena*, from F. Cortesi, unpub. data) to design primers (from Integrated DNA Technologies) to target the one rhodopsin gene (Rh1), two short-wavelength opsin genes (SWS2Ab and SWS2b), and two long-wavelength opsin genes (LWSx1 and LWSx2) present in *A. phalaena*, assuming the same number of opsin genes for *E. lori*. A phenol-chloroform extraction was used to extract DNA from a 116 dph (collected at hatch and reared in the lab) and a wild caught *E. lori* that had been preserved in RNA-later in Belize. A series of PCR reactions were run using the ExTaq™ DNA Polymerase (Takara Bio USA, Mountainview, CA, USA) following the manufacturer's recommendations. PCR products were cleaned (Macherey-Nagel PCR Cleanup Kit) and sequenced at the University of Rhode Island Genomics and Sequencing Center using the BigDye Terminator v3.1 Cycle Sequencing Kit (PE Applied Biosystems [ABI], Foster City, CA, USA) and then trimmed and assembled in Geneious v9.1.5.

Some sequencing results (the short and long wavelength opsins) showed double peaks in the nucleotide signals, which potentially indicated that two genes were being sequenced simultaneously which yielded results with very low consensus. The two sequences were likely very similar and could not be separated by adjusting the PCR conditions or via gel extractions. The short wavelength opsin gene sequences (SWS2Ab and SWS2b) from *A. phalaena* were very similar to each other (only a few base-pairs different), which supported our hypothesis that perhaps the double peaks in the

sequencing results could represent two different shortwave opsin gene sequences. The same situation existed for the longwave sequences (LWSx1 and LWSx2). Therefore, competent cell cloning was used to definitively separate the two similar sequences since the competent cells only pick up one gene copy allowing a single colony with one gene type to be targeted and sequenced. The procedure was as follows:

Day 1

1. Following the kit instructions (Macherey-Nagel Nucleospin Plasmid Easypure), all reagents were thawed on ice and the buffer was vortexed vigorously before use.
2. 3 μ L of PCR product of target sequences (post-cleanup) and 7 μ L ligation solution were put in clean PCR tubes and then mixed by pipetting up and down.
3. Samples were stored overnight at 4°C.

Day 2

4. At the end of the Day 2, agar plates with carbenicillin were pre-warmed at 37°C and a heat block with water in the wells was pre-warmed to 42°C.
5. Agar plates were re-sterilized using 100% ethanol and spread using a flamed spreader bar. Next, 100 μ L of ICMP and 20 μ L of beta galactose was added to the center of the plate and spread very gently, then the plate was placed back in the incubator at 37°C.
6. 2 μ L of thawed PCR product-ligation mixture (from Day 1) and 25 μ L of competent cells (carbenicillin resistant *E. coli*) were added very gently into a

fresh tube and incubated for ~20 minutes on ice, followed by a 45 second heat shock in the 42°C heat block, and then back on ice for 2 minutes.

7. The competent cell mixture was then added and spread onto the previously prepared plate and placed upside down in the incubator overnight (12 hours).

Day 3

8. New plates were prepared following Step 5.
9. White colonies from the competent cell plate were picked using a toothpick and placed on the new plate to provide more space for colonies to grow. Blue colonies were avoided because the color indicated they did not pick up the gene sequence that was added.
10. The new plates were incubated at 37°C overnight (12 hours).

Day 4

11. 2 mL nutrient broth and 1 µL carbenicillin per sample were added into a 10 mL culture tube.
12. Desired colonies were added to the culture tube, sealed, and put on an incubated shaker plate at 37°C overnight.

Day 5

13. Contents of the culture tubes were emptied into 1.5 µL tubes and centrifuged. The supernatant was discarded.
14. The directions from the extraction kit (pGEM-T easy vector system) were followed and samples were submitted for sequencing at the University of Rhode Island Genomics and Sequencing Center using the BigDye Terminator

v3.1 Cycle Sequencing Kit (PE Applied Biosystems [ABI], Foster City, CA, USA) and then trimmed and assembled in Geneious v9.1.5.

Once DNA sequences were obtained for each opsin sequence in *E. lori*, preparation for the RT-qPCR could begin. First, RNA was extracted from specimens so that the relative expression could be determined. DNA gene sequences are always present in an organism, but the amount of RNA of a certain gene sequence indicates how much that gene is being expressed at a certain point in time within the organism. An ontogenetic series of *E. lori* (0 dph [n=38], 6 dph [n=22], 10 dph [n=30], 20 dph [n=9], 21 dph [n=10], 24 dph [n=8], 30 dph [n=7], 58 dph [n=9], 116 dph [n=6] and wild-caught settlers [n=9]) reared in our field lab in Belize was preserved in RNAlater and stored at -20°C for ~12 months. Several months later, RNA was extracted from tissue using a modified protocol for the Qiagen RNeasy Kit as follows:

1. Fish were dissected in RNAlater. For fish for fish < 6 dph whole specimens were used, for fish >6 dph only heads were used.
2. Fish were moved to distilled H₂O, lightly vortexed and then moved to a new tube of distilled H₂O, and vortexed again.
3. Fishes of the same age were split into two or three technical replicates and replicate groups were each moved to a new tube with lysis (RLT) buffer (50-200 µL) and ground with a sterile micropestle.
4. Another 600 µL RLT was added to suspend the tissue.
5. 600-700 µL of the sample was pipetted into Qiashredder filter and the filter was centrifuged at 21,000 rcf for 3 minutes.

6. One volume of ethanol was added to the flow through and mixed and 700 μL of the sample was added to an RNAeasy filter and centrifuged for 30 sec at 11,000 rcf. The flow through was discarded and centrifuging was repeated until the whole sample was spun.
7. 700 μL of buffer RW1 was added to the filter, the sample was centrifuged for 30 sec at 11,000 rcf, and the flow through was discarded.
8. 500 μL of RPE was added, the sample was centrifuged for 30 sec at 11,000 rcf, the flow through was discarded, and this step was repeated.
9. The filter was put in a new tube and centrifuged for 60 sec at 11,000 rcf to remove all remaining ethanol.
10. The filter was put in a new, final collection tube with 25 μL of RNase-free H_2O and centrifuged for 60 sec at 11,000 rcf.
11. Another 25 μL of RNase-free H_2O was added to the tube and centrifuged for 60 sec at 11,000 rcf.
12. Samples were then stored at -80°C for RT-qPCR.

The reverse transcription (RT) reaction translates the extracted RNA into cDNA so that the qPCR light cycler can replicate the cDNA in preparation for the determination of relative expression. The RT reaction (from Carleton, 2012) was as follows:

1. The volume of RNA needed to obtain 0.5 μg of RNA for the RT reaction was calculated.
2. The volume of Master Mix required was calculated and mixed, with 2.5 μL polyT primer and 1.25 μL dNTPs for each sample.

3. Each sample had a total of 15.6 μL of liquid with 3.75 μL of Master Mix, the calculated volume of RNA, and enough water to reach the total volume.
4. The samples were heated to 65°C for 5 minutes and then quenched on ice for 1 min.
5. 5 μL of 5x first-strand buffer (Superscript III kit), 2.5 μL of 0.1 M DTT (Superscript III kit), and 0.65 μL of RNaseOUT was added to each sample and spun down.
6. The samples were kept at room temperature for 2 minutes and then 1.25 μL of Superscript III was added to each sample, bringing the total volume of each sample to 25 μL .
7. The samples were briefly vortexed, spun down, and then kept at room temperature for 10 minutes.
8. Samples were then incubated at 42°C for 50 minutes, made heat-inactive by heating to 70°C for 15 minutes, and then stored at -20°C.

The transcribed cDNA (see above) was then used for qPCR to determine the relative amount of cDNA in the sample, and therefore, the relative expression of that gene. The qPCR reaction (from Carleton, 2012) was as follows:

1. 10 μL of 2x TaqMan universal PCR Buffer (from TaqMan Fast Advanced Master Mix Kit), 3.5 μL of distilled H₂O, and 0.5 μL of cDNA mixture was added to each well of a qPCR plate for each sample and cDNA to be quantified (i.e., five opsins being tested for in each sample = five wells per sample).

2. 2 μ L of forward primer, 2 μ L of reverse primer, and 2 μ L of probe was added to each well for the opsin gene being tested.
3. Each well was mixed and the qPCR plate was sealed with sealing tape and lightly spun down in a centrifuge.
4. The plate was run on the Roche LightCycler® 480 system for at least 40 cycles, with the cycling parameters determined using the primer-probe characteristics. For example, a potential cycling run would be a denaturation step at 95°C for 15 sec, an annealing step at 55°C for 30 sec, and an extension step at 65°C for 1 min.
5. The critical cycle numbers (how many times to run the cycle) were determined for each sample using the second derivative of the standard curve (determined using a serial dilution of desired sequence prior to the current run).
6. Cycle results for each sample (cDNA replication curves) were exported from the Roche system for relative expression analysis.

Statistical Analysis

Morphological data were used to test for two questions: 1) Are the different retinal layers growing proportionately throughout development in *E. lori* (Figure 2.3)? and 2) Is there a change in growth rate of the retinal layers at any point during ontogeny? To address question 1, the values for each layer were standardized for comparison across layers (fish size = $x - \text{mean } x$ and layer thickness = $y / \text{mean } y$). The layer thickness values were log transformed to achieve normality and the slopes were then compared using an Analysis of Covariance (ANCOVA) test (Figure 2.4 and Table 2.1). The model with the best fit was selected based on the R^2 value and the Akaike information criterion

(AIC). The residual values from the model were plotted and tested for normality using a Shapiro-Wilk normality test. To address question 2, a series of regressions were tested for each retinal layer (linear and exponential) and the regression with the highest R^2 value was determined to be the best fit (Table 2.2).

RESULTS

Morphological Analysis of the Ontogeny of the Eye

Overall eye morphology in *E. lori* is similar to that in other teleost fishes studied previously (e.g., *Danio rerio*, *Salmo gairdneri*, and *Poecilia reticulata*, Kunz, 2001). All retinal layers are present at hatch (0 dph) and they increase in thickness over time at varying rates. The ganglion cell layer is the layer most proximal to the lens (Figure 2.1). In small individuals, it is completely in contact with the lens (Figure 2.2A), but by ~20 dph the lens and the retina are not in contact and the circumference of the retina is increasing (Figure 2.2C). The ratio of lens to eye diameter (rostral-caudal diameter of the lens: R-C diameter of the eye) remains constant at 0.40 ± 0.02 SD (Figure 2.2), which is similar to what was observed in the ontogeny of the eye in black bream (Shand et al., 1999b).

The rates at which the thickness of the different retinal layers increase appear to separate into two different groups. Some of the layers (amacrine, bipolar, ganglion and photoreceptive layers) appear to demonstrate linear growth, while the rate of increase in thickness of the remaining layers (inner plexiform, outer plexiform, and pigmented epithelium layers) appears to change through ontogeny (Figure 2.4 and Table 2.2). An ANCOVA indicated that the best model to explain the growth patterns observed in the retina includes an interaction between retinal layer and fish size ($p = 2.2e^{-16}$, Table 2.1). Fish size had a significant effect on retinal layer thickness ($p = 1.91E-07$) and both the pigmented epithelium and inner plexiform layers showed significantly greater changes in

thicknesses than other layers ($p = 0.0436$ and $p = 0.00164$, respectively). The interaction between fish size and retinal layer was significant for all layers except the photoreceptor layer (Table 2.1). The ganglion cell layer thickness had a negative relationship with layer and fish size, indicating that the growth rate of this layer actually decreased as the fish increased in size ($p = 0.00149$).

Molecular Analysis – Opsin Sequences Through Ontogeny

The molecular portion of this study proved to be more challenging than anticipated. The gene sequence for the *E. lori* Rh1 rod opsin was identified from the DNA extraction (Figure 2.4), but the sequences for the long and short wavelength cone opsin genes remained elusive, even after competent cell cloning. The Rh1 gene is commonly used for construction of molecular phylogenies in fishes (Taylor & Hellberg, 2005; Larmuseau et al., 2010) because of its relatively conserved sequence, making it easy to target across wide taxonomic groups (Figure 2.4). Among species, the Rh1 gene has also been tuned to the specific wavelengths that are most prevalent in the environments the species inhabits, without drastically changing the gene sequence (Larmuseau et al., 2010). Within a species, the pairs of short and long wavelength opsin gene sequences (SWS2Ab and SWS2b, LWSx1 and LWSx2, respectively) tend to be very similar to each other (only a difference in a few base-pairs), but there is a high variability in the sequences among species (Figure 2.5). The cloning results in this study yielded three distinct ~800 base-pair sequences. These sequences were then searched for on GenBank, but did not return any known results, not even any bacterial sequences that may have indicated contamination. After reviewing the dissimilarity of the cone opsin

genes sequences of other fishes (Figure 2.5), it was determined that the primers designed to target the opsins of *A. phalaena* in this study may be too dissimilar to target the same genes in *E. lori*. It was concluded that alternative methods (genome sequencing or transcriptomics, see Discussion) should be pursued. With only one nucleotide sequence of the five opsin genes identified in *E. lori*, it was determined that the RT-qPCR portion of the study should be delayed until further methods could elucidate the remaining opsin gene sequences.

DISCUSSION

Elacatinus lori settles on yellow tube sponges and selects these settlement sites visually (Majoris et al., 2018a). It was hypothesized that *E. lori* would have opsins that provide sensitivity to yellow wavelength light (560-590 nm). A few previous studies that assessed the spectral sensitivity of individual or double cones through ontogeny of fishes with pelagic larvae are the basis for this prediction. For instance, Shand (1993) found that the goatfish *Upeneus tragula* demonstrates a dramatic loss of its red-sensing opsin and a shift from longer to shorter wavelength sensitivity (580 nm to 530 nm, roughly yellow to green) of its double cones at settlement. This shift was explained as an adaptation to the significant change in environment from pelagic zone to deeper waters where red light is less available. Losey et al. (2003) were able to target specific rods and cones to test for sensitivity using dissected and prepared eyes in 38 Hawaiian fish species examined using microspectrophotometry. In the current study, previously prepared whole-mount slides were used, so the microspectrophotometric investigation could not be conducted. Instead, the current study looked at the gross morphology of the retina and eye to assess the general visual capabilities of *E. lori* and planned to use molecular data to assess the general spectral sensitivity of the rods and cones.

The visual capabilities of fishes are highly diverse (Losey et al., 2003), with light sensitivity spanning from the ultra-violet (~300 nm, Carleton et al., 2016) to red wavelengths (~750, Shand, 1993). Many recent studies use genomic approaches to explore the great visual diversity of fishes. Two common methods that are used to determine relative expression of opsin sequences are RNA sequencing and RT-qPCR.

RNA sequencing is a process of extracting all of the RNA from a specimen and sequencing it in bulk, then searching through the returned sequences for the desired sequences (Wang et al., 2009), like Rh1 opsin sequences, for example. This method is useful when you get very high RNA yields from the extraction but may also be prohibitively expensive when there are many desired runs (~\$1,000 per run, UC Davis). RT-qPCR is an effective tool even when RNA extraction concentrations are low and running costs are generally lower than RNA sequencing (~\$18 per run, URI; Carleton, 2012). This method targets specific gene sequences using designed primers and probes, but does tend to require some tinkering to achieve optimal running conditions (Costa et al., 2013). The use of both RNA sequencing and RT-qPCR is becoming the most prevalent approach for examining relative expression of known genes (Luehrmann et al., 2018). Both of these methods require the researcher to know the gene sequence they would like to determine the relative expression of. Herein lies the challenge of the current study. Without the confirmation of the DNA sequences, it was not possible to design specific primers and probes for RT-qPCR.

In contrast, DNA sequencing and whole genome sequencing are used to determine a previously undescribed gene sequence. DNA sequencing (used in this study) can be successfully used to identify a gene sequence using a targeted primer, generally when the desired gene sequence is highly similar to other described species (Liu, 2015; Figure 2.5). This allows for accurate primer design and allows for fast and cheap results (~\$5 per run, URI). If a gene has not yet been described in any members of a taxonomic group then it may be unlikely that a designed primer can target it (Figure 2.6). Whole genome sequencing extracts all of the DNA and is then fully sequenced. This method

tends to be very cost and labor intensive since so much sequencing and description accompanies the procedure (~\$10,000 per 8 lanes, Cornell University) but is incredibly useful in learning more about and describing the entire genome of a species. The current study was unable to identify the long and short wavelength opsins in *E. lori* using DNA sequencing and did not have the budget to perform whole genome sequencing. The advancement of genomic tools continues to create opportunities for researchers to explore new questions about gene sequences, gene expression, and gene function. As these genomic tools become more commonplace, morphological studies continue complement our understanding of how the changes in gene sequences directly affect the function of the eye.

This study found that there were observable changes in the morphology of the retina and retinal layers throughout ontogeny of *E. lori*. The eyes of fishes continue to grow throughout life, unlike mammals and birds (Brown, 1957). This is due in part to the addition of new neurons in the retina and increased complexity of connectivity among those neurons (Johns, 1981). The ganglion cell layer was the only layer to have a negative rate of growth during the larval stage in *E. lori*. The ganglion cell layer is the first to be differentiated from the neuroepithelial germinal cells in embryos and the ganglion cells are the first to stop dividing (Sharma & Ungar, 1980). Kock and Reuter (1978) found the ganglion cell layer in *Carassius carassius* to increase in cell number until the eye had reached 7 mm and then there was no more growth in that layer but other layers did continue to grow. The eye size in respect to body size of *Carassius carassius* is similar to what is observed in *E. lori*, with the eye making up about 5-7% of the total body length when ganglion cell layer growth terminated. The overall size of the retina

can also continue to increase through expansion, where the cells spread out to have a lower density but cover a greater amount of space (Johns, 1981). This will increase the surface area the retina covers but will tend to decrease the thickness of each layer. Expansion occurs in older fish once retinal cells are no longer being added. We could not observe this phenomenon in *E. lori* but it may be apparent in larger specimens.

The visual capabilities of a fish may change as the eye and retina grow. For instance, Johns (1981) found that the shape and size of the sensory field does not change during development in goldfish (*Carassius auratus*). Another factor that affects the visual capabilities of a fish is the density of the photoreceptor cells in the retinal mosaic. A greater density of cones leads to greater visual acuity, which tends to be observed in larger fish because they have larger eyes and therefore more cones (Northmore and Dvorak, 1979).

In this study, we observed the position of the lens changed drastically. In day-of-hatch larvae, the lens was in contact with the retina and by 20 dph the lens was no longer in contact with the retina (Figure 2.2). This likely has implications for visual capabilities because the light being focused by the lens would intercept the retina differently upon the lens distance from the retina. This was also observed by Shand et al. (1999b) in *Acanthopagrus butcheri* where the position of the lens was correlated with the development of the accommodatory retractor lentis muscle and focal ratio (focal length:lens radius). In very young fish, the lens is very close to the retina but this is compensated by a very high focal ratio. Throughout development, the lens moves away from the retina, the focal ratio decreases, and the eye gains the ability to accommodate

(Shand et al., 1999b). Fish are likely able to focus on images throughout development, but the focal distance and visual acuity likely vary during this period.

Future studies should pursue alternative methods for identifying the opsin sequences in *E. lori*. It is hypothesized that the likelihood of sensory tuning in this species remains very high given the transition from the pelagic zone to the reef and the specific settlement choices made by larvae (yellow, but not brown sponges). *Elacatinus lori* is a model species for other coral reef fishes with pelagic larvae (D'Aloia et al., 2012; Majoris et al., 2018b; Rickborn & Buston, 2015) and the more that is known about the larval stage of coral reef fishes, the better they can be managed and protected.

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TABLES

Table 2.1 ANCOVA results for the effect of fish size (mm SL) and retinal layer on retinal layer thickness (μm). The multiple R-squared value for this model was 0.8438 and the adjusted R-squared value was 0.8256. The level of significance is indicated by asterisks with * $p \leq 0.05$, ** $p \leq 0.002$, and *** $p \leq 0.001$.

Coefficients	Estimate	Std. Error	t value	P-value
Fish Size	0.0725	0.01305	5.553	1.91E-07 ***
Bipolar Cell Layer	-0.0224	0.05504	-0.41	0.68525
Ganglion Cell Layer	0.0275	0.05504	0.5	0.61822
Inner Plexiform Layer	-0.1777	0.05504	-3.23	0.00164 **
Outer Plexiform Layer	-0.0245	0.05504	-0.45	0.65733
Photoreceptor Layer	0.0054	0.05504	0.099	0.92164
Pigmented Epithelium Layer	-0.1123	0.05504	-2.04	0.0436 *
Fish Size : Bipolar Cell Layer	0.0379	0.01846	2.051	0.04255 *
Fish Size : Ganglion Cell Layer	-0.0601	0.01846	-3.26	0.00149 **
Fish Size : Inner Plexiform Layer	0.1191	0.01846	6.454	2.88E-09 ***
Fish Size : Outer Plexiform Layer	0.0378	0.01846	2.047	0.04299 *
Fish Size : Photoreceptor Layer	0.0120	0.01846	0.65	0.51707
Fish Size : Pigmented Epithelium Layer	0.0880	0.01846	4.766	5.68E-06 ***

Table 2.2 Regression tests outputs of thickness of each retinal layer in *E. lori*. A stronger linear regression may indicate a consistent growth rate through development, while an exponential regression indicates an increasing rate of growth over time. The regression with the highest R^2 value for each layer is bolded.

Retinal Layer	Linear Regression		Exponential Regression	
Amacrine Cell	$y = 0.0902x + 1$	$R^2 = 0.6571$	$y = 0.9554e^{0.0725x}$	$R^2 = 0.5604$
Bipolar Cell	$y = 0.1371x + 1$	$R^2 = 0.9245$	$y = 0.9343e^{0.1103x}$	$R^2 = 0.9181$
Ganglion Cell	$y = 0.0138x + 1$	$R^2 = 0.0421$	$y = 0.982e^{0.0124x}$	$R^2 = 0.0396$
Inner Plexiform	$y = 0.2482x + 1$	$R^2 = 0.8228$	$y = 0.7999e^{0.1916x}$	$R^2 = 0.8794$
Outer Plexiform	$y = 0.141x + 1$	$R^2 = 0.8936$	$y = 0.9323e^{0.1102x}$	$R^2 = 0.9083$
Photoreceptor	$y = 0.0989x + 1$	$R^2 = 0.8876$	$y = 0.9606e^{0.0845x}$	$R^2 = 0.8626$
Pigmented Epithelium	$y = 0.2221x + 1$	$R^2 = 0.8638$	$y = 0.8539e^{0.1604x}$	$R^2 = 0.9033$

FIGURES

Figure 2.1 Eye and retinal structures in *E. lori*. **A)** 0 dph (3mm TL) eye identifying the major structures of the eye identified (cornea, lens, optic nerve and retina) and the axis in which all measurements were taken (yellow dashed line). **B)** High power image of the retina of a wild-caught settler (14 mm SL) with the major retinal layers identified. PE = pigmented epithelium, CM = choroidal melanocytes, Ph = photoreceptors, OP = outer plexiform, BC = bipolar cell, AC = amacrine cell, IP = inner plexiform, and GC = ganglion cell. Scale bars = 50 μ m.

Figure 2.2 Development of the eye, including the retina in *E. lori* using histological material. **A)** 0 dph (3 mm TL), **B)** 10 dph (5 mm SL), **C)** 20 dph (5 mm SL), **D)** 30 dph (8 mm SL), and **E)** Wild-caught settler (14 mm SL). **F)** Close up of retina from white box in A (0 dph). **G)** Close up of retina from black box in E (settler). Scale bar = 50 μm in A, B, C, D, E and 25 μm in F and G.

Figure 2.3 Ontogeny of retinal layers in *E. lori* larvae and settlers. All retinal layers (defined in the Key) are present at hatch (3 mm TL) and increase in thickness throughout development but at different rates except the ganglion cell layer (see Table 2.1). The inner plexiform and pigmented epithelium layers have the greatest rate of growth while the ganglion cell layer has very little growth.

Figure 2.4 Relative growth of each retinal layer in *E. lori*. Thickness measurements were standardized and the mean of the adjusted values and the slope of those values over fish standard length (proxy for time) were calculated. The relative growth rate (slope of layer/mean thickness of layer) is plotted here. All layers increased in thickness over time and the pigmented epithelium and outer plexiform appeared to increase the most rapidly, while the ganglion cell layer had the slowest rate of growth. See Table 2.1 for more information on layer differences.

Figure 2.5 Rod opsin nucleotide sequence alignment of selected goby species. Sequences for ten species of goby were obtained from GenBank, F. Cortesi (*A. phalaena*, pers. comm.), and one sequence (*Elacatinus lori*) generated through PCR. Sequences were aligned using the MAFFT webserver available from EMBL-EBI (<http://www.ebi.ac.uk/Tools/msa/mafft>) and visualized using Genious v.9.15. The rod opsin (Rh1) is commonly used for phylogenetic analysis because of its relatively conserved sequence, which is evident here by the large amount black regions (consensus of nucleotides or sequence similarity). Regions of white indicate dissimilarity between the sequences.

Figure 2.6 Short wavelength opsin (SWS2A) nucleotide sequence alignment of all available goby species. Sequences were obtained from GenBank and F. Cortesi (*A. phalaena*, pers. comm.). Sequences were aligned using the MAFFT webserver available from EMBL-EBI (<http://www.ebi.ac.uk/Tools/msa/mafft>) and visualized using Genious v.9.15. This ~1000 base pair alignment of four sequences indicates regions of conserved bases (black and dark grey) but the overall consensus has a large amount of variation and dissimilarity (white).

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