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Solitary chemosensory cells throughout the life cycle of the sea lamprey, *Petromyzon marinus*

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**SOLITARY CHEMOSENSORY CELLS THROUGHOUT THE LIFE CYCLE OF
THE SEA LAMPREY, *Petromyzon marinus***

By

Tina E. Suntres

A Thesis
Submitted to the Faculty of Graduate Studies
through the Department of Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

2016

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April 29th 2016

Declaration of Co-Authorship

I hereby certify that this thesis incorporates material that is the result of joint research as follows: Chapter 2 and 3 are co-authored with Dr. Réjean Dubuc and Dr. Gheylen Daghfous, under the supervision of Dr. Barbara Zielinski. The contribution of the co-authors is through a collaborative effort in the provision of initial experimental design. Expansion of the initial experimental design to incorporate age class analysis was by the author. The key ideas, data collection, analysis and interpretation were performed by the author with support from Dr. Barbara Zielinski in the form of edits.

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Abstract

The sea lamprey is a basal lineage vertebrate and an invasive species in the Great Lakes. It possess a diffuse chemosensory system with microvillous solitary chemosensory cells (SCCs) located on papillae along the gill pore, oral disc and tail. The objectives of this study were to assess the abundance of SCCs across life stages, and to characterize the innervation and biochemical properties. At all three locations, SCCs were most abundant in the spawning stage compared to earlier life stages, suggesting a role during reproduction. Prominent calretinin and 5-HT labeling show homology to previously identified taste cells and to SCCs in other vertebrates. Labeling for phospholipase C (also seen in mammalian SCCs) suggests that chemosensory signal transduction occurs by an IP_3 mediated cascade. This study suggests that SCC function is important during the end of the sea lamprey life cycle and shows homology between lamprey SCCs and more derived vertebrates.

Dedicated to my family, Steve, Mary, Evan, Samantha, and Benny.

“If I have seen further, it is by standing on the shoulders of giants”

- Sir Isaac Newton

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List of abbreviations used

CR – calretinin

IHC – immunohistochemistry

IR – immunoreactive

PB – phosphate buffer

PBS – phosphate buffered saline

PBS-T – phosphate buffered saline with Triton X-100

PFA – paraformaldehyde

PH - phalloidin

PLC – phospholipase C

SCC – solitary chemosensory cell

SEM –scanning electron microscopy

SV2 – synaptic vesicle protein

TBS-T – tris buffered saline and tween 20

Chapter 1: GENERAL INTRODUCTION

Vertebrates possess three chemosensory cells, olfactory sensory neurons in the olfactory system (Kotrschal, 2000), taste cells grouped forming taste buds in the gustatory system (Finger, 1997), and extra-nasal solitary chemosensory cells (SCCs) which are part of the diffuse chemosensory system (Kotrschal, 1991; Whitear, 1992; Kotrschal, 1996). SCCs were first described in aquatic vertebrates such as the lamprey and in triglids in early nineteenth-century literature (Whitear, 1992) but were more recently characterized by Whitear and Lane in 1983, who found SCCs (also referred to as oligovillous cells) expressed along the skin, gills arches, oral disc and tail of *Lampetra fluviatilis* and *Lampetra planeri*. In mammals SCCs are found in the nasal respiratory epithelium (mice: Finger et al., 2003; Saunders et al., 2014) and along the respiratory tract, larynges, and trachea (cow: Tizzano et al., 2006). These SCCs are classified as a group of apical microvilli that project independently from the surface of the skin (Whitear and Lane 1983; Kotrschal, 1997). Each SCC connects to a basolateral membrane that is densely supplied with nerve endings indicating these SCCs can transmit their signals to higher brain structures (Kinnamon, 2012).

Mammalian solitary chemosensory cells

The exact role of this sensory system in fish is still unknown; it has been postulated that they are involved in discriminating conspecifics and alert functions in rocklings (Teleostei, Gadidae: *Ciliata mustela*) as SCCs are present on their undulating dorsal tail fin respond to fish body mucus (Peters et al., 1991; Finger 1997), foraging and food related behaviours in the sea robin (Teleostei, Triglidae: *Prionotus carolinus*) where SCCs are on their free pectoral-fin rays and respond to nutritionally relevant substances

such as amino acids (Kotrschal, 1995, 1996; Finger, 1997), or possibly for other behaviours, such as mating (Finger, 1997). SCCs have been found in many fish species (Finger 1997; Hansen et al., 2014; Kotrschal, 1991, 1995; Whietear, 1992). They are also present in alligators nasal cavities (Hansen, 2007), and in the respiratory systems of mammals (Sbarbati and Osculati, 2003). Multiple studies have helped to elucidate SCCs and their chemosensory abilities, but more research is still needed on behaviour and development to understand the function and purpose of this diffuse chemosensory system (Whietear, 1992).

In mammals, SCC activation has been identified by Saunders et al., in 2014, where they found that SCCs present in the nasal cavity of mice respond to bitter irritants to induce an inflammatory response (Fig. 1, Saunders et al., 2014 Fig. 4). Bitter irritants will bind to g-protein coupled receptors on the membrane and activate inositol trisphosphate (IP_3) through a phospholipase C mediated cascade. The IP_3 binds to its type 3 receptor (IP_3R3) to mediate the release of calcium from intracellular storage in the endoplasmic reticulum. This calcium influx signals TRPM5 channels to open, leading to depolarization and acetylcholine release onto nociceptive fibers. Substance P is released from these fibers onto blood vessels to cause an inflammatory response. These mammalian respiratory SCCs have also been shown to be innervated by the trigeminal nerve and monitor for potential toxins entering the air way to initiate protective reflexes such as sneezing (Finger et al., 2003).

Taste cells and solitary chemosensory cells

SCCs share many characteristics and signaling pathways with taste cells from the gustatory system (Finger, 1997; Sbarbati et al. 2005; Kinnamon 2012). (Fig. 2, Finger 2007 Fig. 5). They are both secondary receptor cells, where their synapse located in the peripheral nervous system (Finger, 1997) (in contrast to olfactory sensory neurons that are primary receptor cells with their synapse in the olfactory bulb of the brain), and in the lamprey, oral disc SCCs and taste cells are both innervated by the glossopharyngeal or vagus nerves (Daghfous et al., 2015; Finger, 1997). Taste and SCCs both show apical microvilli (Fig. 2, Finger, 2007), while taste cells are grouped forming buds, SCCs are individually scattered throughout the epithelial surface (Whitaker, 1992; Finger, 1997). In the lamprey, both are immunoreactive for calretinin (Barreiro-Iglesias et al., 2008; Hansen et al., 2014), a calcium binding protein involved in controlling intracellular calcium storage needed for neurotransmission (Diaz-Regueira et al., 2000). Mouse taste cells use ATP as their neurotransmitter (Finger et al., 2005). While the neurotransmitter in fish SCCs is currently unknown (Whitaker, 1992) mammalian SCCs release acetylcholine (Saunders et al., 2014). SCCs are present along the head and body during the hatching of zebrafish eggs, before taste cells which appear 2 days later (Kotrschal et al., 1997), and this has led some to postulate that taste buds evolved from SCCs (Kotrschal et al., 1997; Finger, 1997). Adult lamprey pharyngeal taste buds respond to classical taste stimuli (sweet, bitter, salty, sour) (Baatrup and Doving, 1985A), whereas oral disc SCCs did not, instead they responded to sialic acid (found in trout body mucus) (Baatrup and Doving, 1985B), indicating that these chemosensory cells have evolved

different functional chemoreception spectrums based on their distributions along the body (Kotrschal, 1992).

The life cycle of the Sea lamprey

The Sea lamprey, *Petromyzon marinus*, is basal lineage vertebrate and an invasive species in the Great Lakes that parasitically feeds on the blood of its prey (Applegate, 1950). It has a phasic life cycle (Fig. 3, GLFC) starting in sediments as filter feeding larvae (Moore and Mallatt, 1980) which undergoes a metamorphosis stage into a juvenile parasitic stage that feeds in the deep lakes on approximately 40 lbs in a 12-18 month period which greatly affects the native fish populations (Johnson et al., 2015). As adults, they migrate at the end of their life cycle from the deep lakes where they feed, to the streams and rivers where spawning occurs. During upstream migration, pheromones released by the larvae located in spawning streams attract migrating adults (Meckley et al, 2012). Once sexually mature males arrive at nesting sites, these males release sex pheromones to attract ovulating females (Walaszczyk et al., 2013). Chemosensory systems are necessary for the life history behaviours and ecosystem functioning of lamprey within their environment.

Thesis objectives

Studying SCC distribution and neuroanatomy in the sea lamprey will help to shed light on the function of the diffuse chemosensory system. Combining microscopy visualization techniques with physiology will be needed to know how this system functions.

In the first data chapter of this thesis, the distribution of SCCs present around the gill pore of larval, metamorphic, juvenile, pre-ovulated and pre-spermiated adult migrating stage and ovulated and spermiated spawning adult stage sea lamprey were examined using scanning electron microscopy and immunohistochemistry techniques. The hypothesis of this chapter was that the stage that possessed the most abundant amounts of observed SCCs would also utilize the detection capabilities the most, and therefore infer a function of the diffuse chemosensory system in the sea lamprey, as each stage has very different life history behaviours, ranging from filter feeding, to active parasitically feeding, and to migrating and spawning while not feeding. Immunohistochemistry was also used to investigate the neuroanatomy and biochemistry of lamprey SCCs in comparison to mammalian SCCs, as well as to the lamprey taste system. This allowed for the characterization and comparison of traits to be identified, such as the presence of microvilli, innervation patterns and transduction pathways.

The second data chapter of this thesis compares the SCC (oligovillous) distribution of larval, metamorphic, juvenile, pre-ovulated and pre-spermiated adult migrating stage and ovulated and spermiated spawning adult stage lamprey SCCs across oral disc and tail papillae. Again, the life history stage that possessed the most SCCs would infer a function of the diffuse chemosensory system. Also, as SCCs occur on papillae present in these separate locations, there may be different function based on the spatial relationship of SCCs and their distribution.

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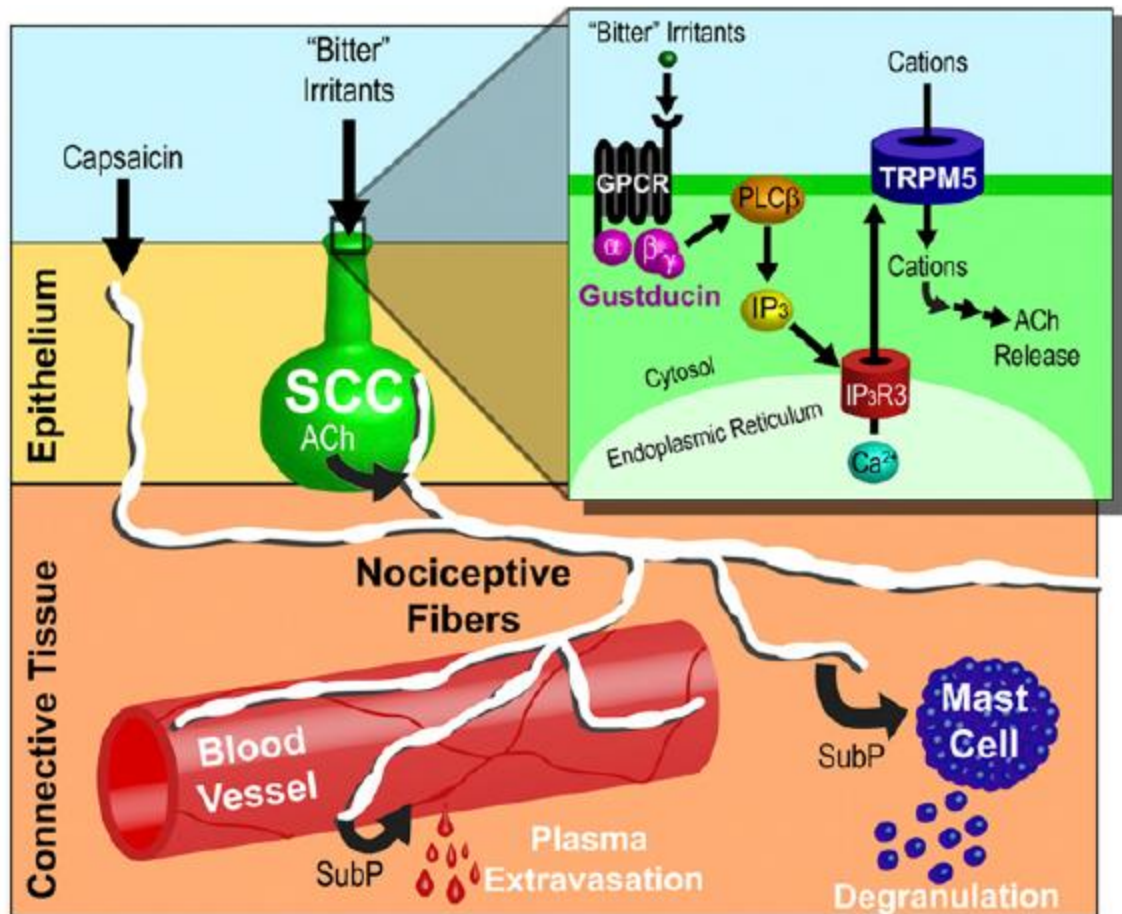


Figure 1.1 SCC transduction pathway in mice. Image from Saunders et al., 2014. SCCs present in the nasal respiratory epithelium detect bitter irritants from the airways and release acetylcholine onto nociceptive nerve fibers that release substance P onto NK₁ receptors on blood vessels present in the connective tissue layers initiating an inflammatory response. Capsaicin also initiates the same inflammatory pathway of nociceptive fibers and blood vessels.

(Inset) G-protein coupled receptors are stimulated by bitter irritants and activate the $\beta\gamma$ -subunit of α -gustducin protein. This catalyzes the formation of inositol triphosphate (IP₃) through a phospholipase C mediated cascade. IP₃ then binds to its type 3 receptor (IP₃R3) to release calcium from the endoplasmic reticulum. This influx of calcium activates

TRMP5 receptors on the membrane to allow cations to enter and the SCC will depolarize and release acetylcholine.

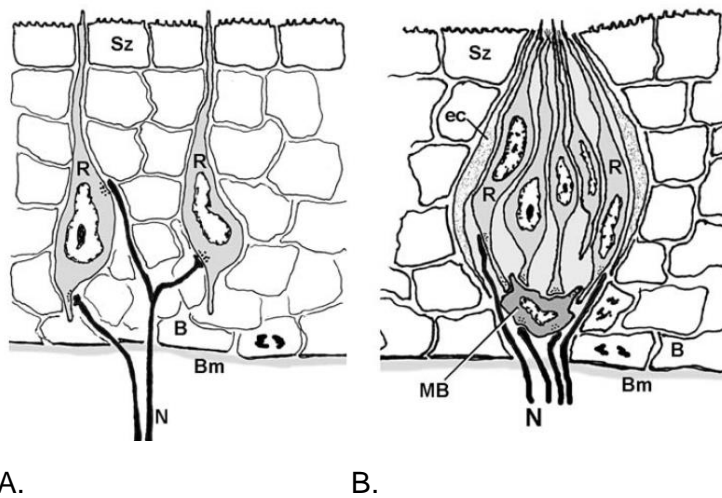


Figure 1.2 Drawing of a comparison of a typical teleost SCCs (a) and taste buds (b).
(Image modified from Finger, 2007).

A. Monovillous SCCs are individually present within the epithelium, and are contacted by branching nerve fibers.

B. Multiple cell types are present within one taste bud with an open pore to the external environment. Nerve fibers approach the base of the taste bud.

(B, basal cell; Bm, basal lamina (basement membrane); ec, edge cell; MB, Merkel-like basal cell; N, nerve fiber; Np, nerve plexus; R, receptor cell; Sz, mucous cell; Sg, glandular supporting cell; St, type II supporting cell).

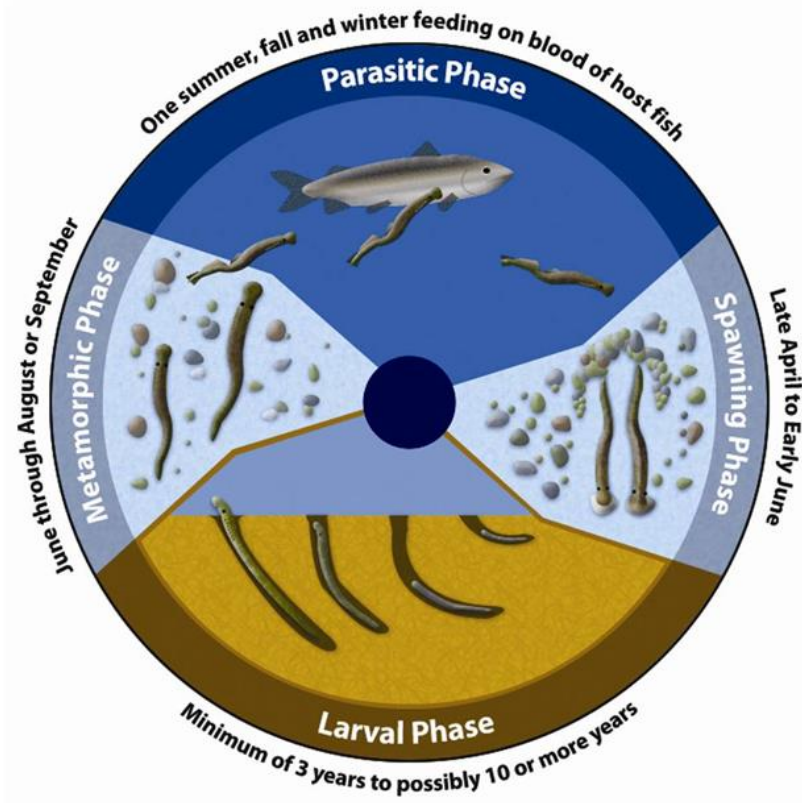


Figure 1.3 The sea lamprey, *Petromyzon marinus* life cycle. The sea lamprey begins its life in the sand as a filter feeder. It then undergoes metamorphosis and migrates from streams and tributaries to the deep lakes. They are then in a juvenile parasitic stage where the lampreys are feeding on other fish species. After this stage they stop feeding and start to become sexually mature as they migrate back to streams to lay their own eggs in one spawning event.

(Image from the Great Lakes Fishery Commission website,
http://www.glfcc.org/sealamp/images/sea_lamprey_life_cycle.jpg)

Chapter 2: LIFE HISTORY, BIOCHEMICAL AND NEURAL TRAITS OF GILL PORE SOLITARY CHEMOSENSORY CELLS IN THE SEA LAMPREY

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CHAPTER 2 SUMMARY

Solitary chemosensory cells (SCCs) innervated by nerve fibers are located near respiratory areas in many vertebrates. While SCCs have been observed on papillae that line the posterior surface of gill pores in lampreys, little is known regarding the development of these structures during life cycle, innervation patterns or the biochemical traits of SCCs in this basal vertebrate which is an invasive species in the Laurentian Great Lakes. In this study, we found that papillae were absent and that SCCs were sparse in the larval stage and during metamorphosis; few SCCs were seen on small nub-like papillae during the juvenile stage but SCCs were abundant on prominent papillae during the adult stages. Calretinin and 5-HT labeling of these SCCs, and basal innervation by acetylated-tubulin immunoreactive fibers showed homology to previously identified taste cells in the lamprey pharynx and to SCCs in other vertebrates. Immunolabeling for phospholipase C suggests that chemosensory signal transduction occurs by an IP₃ mediated cascade. This

study infers that SCC function is important during the adult stage of the sea lamprey life cycle and shows homology between the SCCs lamprey and more derived vertebrates.

Key words: solitary chemosensory cell, anatomy, lamprey, development

INTRODUCTION

The specialized epithelial chemosensors known as solitary chemosensory cells (SCCs) are located on the skin of aquatic vertebrates (Whitear, 1992), including the body, oral cavity and gills of fish (Kotrschal et al., 1996; Finger, 1997), in the nasal cavity of amphibians (Hansen, 2007) and in mammalian respiratory epithelium (rodent: Finger, 2003; cow: Tizano, 2006). In mammals, bitter irritants evoke changes in respiratory rates by activating trigeminal protective reflexes (Finger et al., 2003). In teleost fish such as sea robins (Teleostei, Triglidae: *Prionotus carolinus*), SCCs aid in detecting feeding cues and promoting foraging (Bardach and Case, 1965; Finger, 1982; Kotrschal, 1995, 1996); and in rocklings (Teleostei, Gadidae: *Ciliata mustela*) SCCs sample the water for fish body mucus and bile as a method of detecting prey or predators (Bardach and Case, 1965; Silver and Finger, 1984). Extracellular recordings from individual lamprey gill papillae showed multi-unitary action potentials in response to amino acids and dead trout water washings (Daghfous, 2014).

The different stages of the sea lamprey life cycle are associated with specific behaviours. It is expected that SCC abundance correlates to the importance of these cells during a particular stage. Several changes occur during the life cycle; the larval stage is a benthic filter feeder (Moore and Mallat, 1980), and then undergoes a metamorphic stage into a juvenile stage that feeds on the blood and tissues of fishes for 12-18 months

(Hardisty, 2006). The lampreys then cease feeding and migrate up streams and rivers as pre-ovulated and pre-spermiated adults into spawning grounds for reproduction as fully mature ovulated and spermiated adults (Manion and Mclane, 1971). Sexually mature males arrive at nesting sites first to construct nests, and release sex pheromones to attract ovulating females (Walaszczyk et al., 2013). While pheromones (Li et al., 1995) and migratory behavioral responses (Applegate, 1950; Bjerselius et al., 2000; Fine et al., 2004; Johnson et al., 2009) have been well characterized, the diffuse chemosensory system is still poorly understood in lampreys.

In lampreys, SCCs are located on papillae protruding along the oral disc, nasal pore, gill pores and dorsal tail fins (Whitear and Lane, 1983) and are similar to the cells that are grouped in taste buds on the pharynx and gill arches (Mallat, 1979). Lamprey SCCs (also referred to as oligovillous cells) are characterized by multiple microvilli projecting from the apical surface of the skin (Whitear, 1983B). Calretinin has been shown to localize in teleost SCCs (Hansen et al., 2014) and may also localize in lamprey SCCs, as this calcium binding protein has been shown in lamprey pharyngeal taste buds. Inositol triphosphate (IP_3) is a second messenger prevalent in mammalian SCCs (Saunders, 2014), the formation of IP_3 is catalyzed through a phospholipase C-mediated cascade and but it is not known whether lamprey SCCs utilize this same pathway. Vertebrate SCCs are embedded in the epidermis and are innervated by spinal and cranial nerves (Dagfous, 2015; Finger, 1997; Hansen et al., 2014), single nerve fibers approach and are adjacent to the base of the SCCs (Whitear, 1992). Neural fibers that are α -tubulin-immunoreactive approach the base of lamprey taste buds with serotonergic fibers present below the taste buds in the basal lamina as well as in lamprey taste cells

themselves (Barreiro-Iglesias et al., 2008C). This labeling has not been explored within lamprey SCCs.

The aim of the present study was to gain understanding of the evolution and function of the diffuse chemosensory system. Investigating the abundance of brachial SCCs during the phasic life cycle of the sea lamprey, with feeding taking place during a separate phase from reproduction, will shed light on the importance of the SCCs. Neurochemical characterization will improve knowledge of potential signal transduction and neurotransmission strategies utilized by the SCCs in this basal vertebrate.

MATERIALS AND METHODS

Experimental animals and tissue preparation

Sea lamprey, *Petromyzon marinus* larvae (n = 2), metamorphic (n = 4), juvenile (n = 4), pre-ovulated and pre-spermiated adult stage (n= 24) and sexually mature ovulated and spermiated spawning adult stage fish (n = 30) were caught from Lake Huron and the surrounding tributaries by Hammond Bay Research Station, MI and transported to the University of Windsor Biology Building. Each animal's weight and length were measured and recorded (See Table 1, Appendix B). The Lamprey were euthanized by anaesthetic overdose (1 g/L MS-222), and dissected to collect both sides of seven gill pores. All protocols were approved by the Canadian Counsel for Animal Care. The right side of each animal was drop fixed in 5% gluteraldehyde fixative for scanning electron microscopy (SEM) and the left side of each animal was drop fixed in 4% paraformaldehyde fixative for less than 24 hours for immunohistochemistry.

Migrating pre-ovulated and pre-spermiated adult male and female sea lampreys were captured in traps from Lake Huron tributaries (United States Fish and Wildlife Service, Marquette, Michigan, USA) and held in 1000 L tanks supplied with Lake Huron water at ambient temperature at United States Geological Survey, Great Lakes Science Center, Hammond Bay Biological Station, Millersburg, Michigan, USA. They were brought to the University of Windsor Animal Quarters on May 13th, 2015. Sexually mature (ovulated and spermiated) spawning adult lamprey were captured directly from spawning nests by hand from the Ocqueoc River on June 8th 2015 and brought to the University of Windsor on June 9th 2015. Both groups were processed for experimental use.

Tissue preparation for scanning electron microscopy

Lamprey gill pores were fixed in a 5% glutaraldehyde 0.2M sodium cacodylate solution. Tissue samples were washed with 0.1M sodium cacodylate buffer then treated with 2% osmium tetroxide in 0.1M sodium cacodylate buffer, and an ethanol dehydration series was completed. The samples were taken to the Integrated Microscopy Biotron Facility, Western University to undergo critical point drying and gold sputter coating. All SEM micrographs were taken on a FEI Quanta 200 FEG environmental scanning electron microscope at the Great Lakes Institute for Environmental Research at the University of Windsor, ON.

Counts of SCCs on gill papillae of sexually immature migrating and spawning adult lampreys

The 2nd most rostral gill pore was assessed by SEM from 3 pre-spermiated males, 3 pre-ovulated females, 3 spermiated spawning males and 3 ovulated spawning female

lampreys since a previous study reported similar amounts of papillae on gill pores 2-6 of the 7 gills (Beamish, 2010). Images were standardized by rotating center gill pore papillae to in a horizontal plane in the SEM, and magnified to 1500X. These were then centered with the tip of the papilla at the edge of the field of view then magnified to 3000X. This produced the same image area and had a set distance from the SEM detector to standardize the size of the area examined to (45 μm x 45 μm = 2025 μm^2). The number of SCCs was counted from three different gill papillae for each specimen. Counts were performed with no sample information to minimize bias.

Tissue preparation for immunohistochemistry

Samples were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). Excess tissue surrounding the areas of interest then drop fixed in fresh 4% paraformaldehyde for 1-3 hours. Tissue was then transferred to 20% and 30% sucrose solutions for cryosectioning. Samples were sectioned on a Leica CM3050S cryostat. The gill pores were serial sectioned in 20 μm horizontal sections or in 20 μm cross sections. All tissue was thaw-mounted onto Superfrost Plus slides (Fisher Scientific).

Immunohistochemistry

Slides were washed with phosphate buffered saline solution (PBS) with 0.1% triton X-100 (PBS-T) over 2 hours with the PBS-T wash solution replaced every 30 minutes. PBS plus 0.1% sodium azide was used as a preservative against bacterial growth in all antibody keepers. All steps in the protocol were completed covered on a shaker at 4°C. Slides were incubated in a 5% goat serum (Sigma, G9023) in PBS-T for 2 hours then transferred into the primary antibody (Table 1) for 3 days, monoclonal acetylated tubulin (1:1000, Sigma, T7451), rabbit polyclonal anti-calretinin (1:1000, Swant,

7699/3), polyclonal anti-phospholipase C 140 (1:1000, kind donation from Dr. Jane Mitchell, University of Toronto), mouse monoclonal anti-SV2 (1:500, the SV2 purified synaptic vesicle antibody, developed by Buckley, K.M., Harvard Medical School, was obtained from the Developmental Studies Hybridoma Bank, created by the NICHD of the NIH and maintained at The University of Iowa, Department of Biology, Iowa City, IA 52242), polyclonal anti-5-HT (1:5000, Immunostar 20080). A 5-HT protocol was followed from Frontini et al. (2003). Wash steps were completed in 5 cycles of 0.1M PBS on a shaker for 30 minutes. Slides were then transferred to a secondary antibody for 1-3 days, monoclonal anti mouse Alexa Fluor 488 (1:100, Life Technologies A11001) or polyclonal anti rabbit Alexa Fluor 568 (1:250, Life Technologies A11011). A phalloidin label was also used (Life Technologies, Alexa 488 A12372 or Alexa 568 A12380) diluted 5 μ L in 400 μ L PBS, applied directly to slides for 30 minutes then washed. Finally slides were washed according to the above cycle and coverslipped with Vectashield Mounting Medium. The slides then dried overnight and sealed with nail polish.

Western blotting - Adult sea lamprey (n = 4) were anesthetized with 0.3g/L MS-222 and dissected in Ringers solution to remove gill pores and brain tissue. Papillae from the gills were dissected out. The tissue samples were homogenized at 4°C in 6X the volume of tissue in modified RIPA buffer (Villar-Cheda, 2006), (50 mM Tris-HCl, 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonylfluoride, 1% Triton X-100, 0.1% sodiumdodecylsulfate, 5 μ g/ml aprotinin, pH 7.4) and left on ice for 20 min for lysis. The homogenate was centrifuged at 20,000 \times g at 4 °C for 20 min, and the supernatant collected. Samples (40 μ g) were loaded on 12 % (Calretinin) or 8% (Phospholipase C) acrylamide gels, run at 1 hour at 150volts, resolved by SDS-PAGE and then

electroblotted at 111 volts for 1 hour (Calretinin) or 1.5 hours (Phospholipase C) onto 0.33 nitrocellulose membrane (Biorad). Non-specific binding was blocked with a 5% milk powdered non-fat milk dissolved in tris buffered saline and tween 20 solution (TBS-T) for 1 hour. After blocking, the membranes were incubated in rabbit polyclonal anti-calretinin (Swant) and anti-phospholipase C (kind donation from Dr. Jane Mitchell, University of Toronto) diluted 10:10 000 in TBS-T overnight. The membranes were rinsed in TBS-T (3x 5 min) then incubated in a mouse anti rabbit HRP-conjugated antibody diluted at 1:10 000 in TBST (Santa Cruz), They were rinsed again in TBS-T (3x 5 min), and then imaged on an Alpha – Innotech imaging system. Page Ruler Prestained protein ladder (Thermo-Scientific) was used as a molecular weight marker.

Antibody characterization

The specificity of the antibodies used (see table 1 for information) were all first tested by the suppliers. As a control for non-specific labeling, the primary or secondary antibodies were omitted from the staining procedures for all tested antibodies.

Calretinin has been previously reported in lamprey taste cells (Barreiro-Iglesias et al., 2008), and in SCCs of Big headed carp (Hansen et al., 2014). Western blots analysis yielded appropriate protein sizes (Fig.1) for calretinin (29kDa) (Villar-Cheda, 2006, Barreiro-Iglesias et al., 2008C) using lamprey brain as it is important for regulating intracellular calcium storage at synapses (Diaz-Regueira et al., 2000). A phospholipase C 140 western blot protocol was modified from Mobley et al. (2007) and yielded appropriate protein sizes (140kDa) in both lamprey gill tissue and squid optic lobe (kind donation from Dr. Mary Baum and Dr. Dan Morse University of California at Santa Barbara) which was used as a positive control as this phospholipase C 140 antibody was

previously shown to be immunoreactive in squid (*Loligo*) chemoreceptors (Mobley et al., 2007).

The acetylated tubulin antibody was used as a general probe for neuronal tissue. It was previously used for labeling sea lamprey neural fibers by Zaidi et al. (1998), Frontini et al. (2003) and Barreiro-Iglesias et al., (2008, 2009). It has been shown to label acetylated α -tubulins in multiple species by the supplier (Sigma). Brain tissue sections were used as a positive control for labeling. The SV2 antibody was obtained from the Developmental Studies Hybridoma Bank to detect axon terminals and fibers. It positively cross reacts in multiple species, including fish. It is also reported by the Hybridoma Bank that it recognizes all three isoforms of synaptic vesicles (SV2A, SV2B, and SV2C). The 5-HT (serotonin) antibody was used to label serotonergic fibers and cells previously tested by western blot in lamprey tissue (Villar-Cervino et al., 2006), and was previously used to label lamprey taste cells (Barreiro Iglesias et al., 2008). Brain tissue sections were again used as positive controls for serotonergic nerve labeling. PreadSORption control experiments were performed using diluted antiserum preadsorbed for 1 hour at room temperature then overnight at 4°C with 5-HT antigen (5mg) (Sigma). No immunostaining was detected (See Fig 2, Appendix C).

Microscopy

Photomicrographs of slides were taken on a Nikon Eclipse 800 epifluorescence microscope, or on an Olympus Fluoview FV1000 (Fluoview version 2.1C) confocal laser microscope, Adobe Photoshop CS6 and ImageJ were used to adjust for brightness and contrast levels.

RESULTS

Gill pores from larval, metamorphic, juvenile and adult stage lampreys were examined for the presence of SCCs by SEM. In accordance with Whitear and Lane's (1983) classification of lamprey oligovillous cells, the SCCs were identified on papillae by their protruding tufts of microvilli. Gill papillae were absent during the larval stage examined and a single SCC was found on the flat posterior surface from the second most rostral gill pore (Fig. 2D). During the metamorphic stage, the posterior surface of the gill pore was slightly undulated (Fig. 2F) with few SCCs (Fig. 2G). The papillae were developed and SCCs were prominent in the juvenile and the adult stage, including migrating (pre-ovulatory females and pre-spermiated males) and spawning (ovulatory females and spermiating males) adults (Fig. 2H-V). A central process is present in the center of the gill pore, that did not possess any SCCs and it is thought that it may play a role in opening and closing the external branchiopore (Beamish, 2010). The number of SCCs in a representative papillar area (45 μm x 45 μm) (Table 2) was greater in spawning adults (ovulated/spermiated) than in juveniles or preovulated/prespermiated adults ($p \leq 0.001$; Fig. 3A). A post hoc Tukey's test also showed no difference in densely packed SCCs along the side of brachial pore papillae observed between males and females for both preovulated/prespermiated ($p = 0.44$) and spawning (ovulated/spermiated) ($p = 0.52$) life stages (Fig. 3A). Overall, a trend was seen that ovulated/spermiated adult lamprey possessed more densely packed SCCs along the side of gill pore papillae observed than pre-ovulatory/pre-spermiating adults, and that females have more SCCs than males.

Biochemical characterization of SCCs

Phalloidin labeling of actin was useful for localizing the SCCs in sectioned preparations. Phalloidin labeling was seen in SCC microvilli (Fig. 4 A, B) and around the cell membranes the oval-shaped SCCs (Fig. 4 C, D).

Double labeling with phalloidin showed that the SCCs were immunoreactive to phospholipase C, calretinin and to 5-HT (Fig. 5). In these preparations, every microvillar cell (labeled with phalloidin) was immunoreactive to phospholipase C, calretinin or to 5-HT. Because of this similar patterning and cell shape, it is possible that phospholipase C, calretinin and 5-HT co-localize in SCCs.

Spatial relationship between nerve fibers and the SCCs

Acetylated tubulin immunoreactive fibers were present in the lamina propria underlying the papillae and reached the apex of the papillae (Fig. 6A, B). In the lamina propria some fibers double labeled for 5-HT and acetylated tubulin and some were single labeled for 5-HT or for acetylated tubulin (Fig. 6A, B). Although 5-HT-ir fibers were observed entering the base of the papillae, these fibers were not seen towards the papillar apex nor in the epithelium (Fig. 6 A, B). In acetylated tubulin, calretinin (or phospholipase C) double-labeled preparations (Fig. 7A,B), it was clear that acetylated tubulin-IR fibers extended into the papillar lamina propria and reached the SCCs within the epithelium, sometimes encircling the base of SCCs (Fig. 7 A). The SCCs themselves were not acetylated tubulin or SV2-IR (Fig. 8). The SV2 labeling was confined to fibers within the lamina propria and did not enter the epithelium (Fig. 8).

DISCUSSION

This study has shown that gill SCCs in lampreys are most abundant during the adult stage, and that these cells share biochemical properties and innervation similar to SCCs in more derived vertebrates and to lamprey taste buds.

The morphology of the SCCs on gill pore papillae matched cells previously characterized in lamprey by Whitear and Lane (1983B). Abundant SCCs in the adult stage of the life cycle compared to larval, metamorphic, and juvenile stage lamprey reflects that found in zebrafish, where SCC density increased throughout its development to adulthood (Kotrschal, 1997). In contrast to the zebrafish which feeds throughout its life, the adult stage of the sea lampreys does not feed. During this stage, the lampreys migrate upstream (pre-ovulating/pre-spermiating), select a spawning location, construct a nest from gravel, and spawn (adult ovulating/ spermiating) (Applegate, 1950). We hypothesize that the SCCs are important for collecting sensory information during these activities. Length and/or total body size may also be correlated to SCC amount, as smaller collected juvenile lamprey possessed very few SCCs compared to the abundant SCCs that the larger migrating lamprey possessed. It should also be noted that both pre-ovulated/pre-spermiated and spawning stage lamprey are not feeding (Manion and Mclane, 1971) and therefore are investing metabolic energy into this diffuse chemosensory system which infers a benefit during the adult stage.

The function of papillae located on the gill pore may be to minimize damage to gill filaments by reducing debris entering the gill pore as they face the flow of water through the gill pores (Beamish, 2010). It has also been postulated by Beamish (2010) that papillae may be a secondary sexual characteristic (a trait that appears during sexual

maturity separate from that of the reproductive system that may infer fitness) because of this increase in size prior to spawning. Male spawning sea lamprey possess another secondary sexual characteristic known as a “rope”, a swollen dorsal ridge (Johnson et al., 2014) that is composed of thermogenic adipose tissue (Chung-Davidson et al., 2013). In the presence of a sexually mature female, males will generate heat at the expense of large amounts of energy into this sexual advertisement. Contact between the female urogenital pore which has been shown to possess SCCs (Whitear and Lane, 1983B) and the rope tissue may play a role in reproduction (Chung-Davidson et al., 2013). We found that females had slightly more gill pore SCCs than males (regardless of sexual maturity stage). Another gill pore-related sexual dimorphism was previously found by Pickering in 1977, where he examined gill tissues of spawning stage lamprey and found that males had more type 1 “male glandular” cells than females which only possessed type 2 cells suggesting a difference between sexes in ion-regulation during spawning. Spermiating male gills have also been shown to be the site of sex pheromone release that will attract ovulating females (Siefkes et al., 2003).

The abundant SCCs in adult stages may be beneficial for choosing a nest site and to collect more sensory information about spawning locations. Males arrive at the breeding grounds before females (Applegate, 1950) and collect rocks to build a nest. Recently it has been shown that SCCs respond to dead trout water washings in electrophysiology recording experiments (Daghfous, 2015), which may indicate that this diffuse chemosensory system is used as a method of irritant detection as SCCs have been shown in lamprey to respond to mucus, acids, and bitter irritants (Baatrup and Doving, 1985B; Daghfous, 2015; Finger, 1997). While mammalian SCCs function to detect

irritants including immune response stimuli (Finger et al., 2003, Saunders et al., 2014), lamprey would benefit from detecting irritants, toxins and decaying material by avoiding these locations for poor egg development (Silva et al., 2015). The diffuse chemical system may function as a “push” behaviour by directing lamprey away from inappropriate spawning sites for propagating the next generation, rather than a “pull”, like olfactory (pheromones) mediated locomotor behaviour (Daghfous et al., 2012, Derjean et al., 2010). As visual information is easily obstructed by turbidity and vegetation, the lampreys may benefit from collecting diverse chemosensory information may at this stage (Wansenbock et al., 1996). It has been shown that vision plays no role in upstream migration of adult sea lampreys during blinding experiments (Binder and McDonald, 2007), as most of their migration occurs as nocturnal movements with light avoidance behaviours during the day (Applegate, 1950; Johnson et al., 2014), therefore chemosensory systems must guide the lamprey to the appropriate spawning location during migration (Binder and McDonald, 2007).

SCCs were identified by the apical microvilli seen by scanning electron microscopy, and in sectioned tissue by phalloidin labeling of actin filaments abundant in microvilli and by the tall oval cell body shape that matched that described previously shown in transmission electron microscopy preparations (Whitear and Lane, 1983B). The localization of phospholipase C to microvillar cells (Fig. 5A) further supports the identity of these cells as SCCs. Mice SCCs have been shown to utilize phospholipase C to initiate a cascade that activates an IP_3 mediated transduction pathway (Saunders, et al., 2014). This lamprey localization is the first report of phospholipase C in non-mammalian SCCs and suggests that the SCCs also use an IP_3 mediated transduction pathway. This

chemosensory transduction cascade has also been identified in olfactory sensory neurons in squid (Mobley, 2007), teleosts (Hansen et al., 2003; Laframboise and Zielinski, 2011), and in mammalian vomeronasal sensory neurons (Szebenyi et al., 2014; Hegg et al., 2010), indicating a conserved chemosensory transduction pathway. In taste cells, phospholipase C mediates the release of calretinin from intracellular stores to open cellular channels (Kinnamon, 2012). The presence of calretinin-immunoreactive cells matches that of the lamprey taste cells (Barreiro-Iglesias et al., 2008C, 2010), and of SCCs in other teleost fish (Germana, 2007, Hansen et al., 2014). Calretinin is important for neurotransmission of signals by controlling intracellular calcium concentrations (Diaz-Regueira et al., 2000; Levanti et al., 2008). Prominent calretinin labeling of gill SCCs shows homology to previously identified taste cells in the lamprey pharynx and to SCCs in other vertebrates (Barreiro-Iglesias et al., 2008C; Hansen et al., 2014).

The presence of 5-HT labeling within SCCs and fibers innervating papillae but not approaching SCCs directly matches previous reporting of 5-HT labeling in the lamprey peripheral taste buds (Barreiro-Iglesias et al., 2008C). Calretinin may modulate the release of serotonin (5-HT) by regulating calcium influx of activated chemosensory cells (Evans et al., 2000; Barreiro-Iglesias et al., 2010). The role of 5-HT in these cells is unknown, but may affect the perception of SCC sensory signals as it is thought to play a role in neurotransmission of taste buds to neurons (Larson et al., 2015).

Acetylated tubulin labeled nerve fibers extended from the base of the papilla and approached the basolateral region of the SCCs. This organization matched previous transmission electron microscopy, which showed the profiles of neurites adjacent in the basolateral regions of the SCCs (Whitaker 1983B, 1992). This innervation pattern also

occurs in the taste system where fibers approach but do not directly enter the taste buds (Barreiro-Iglesias et al., 2008C). Gill pores are innervated by the glossopharyngeal and vagal nerves (Daghfous, 2015) and project into the brain via the trigeminal nerve, which is conserved across teleosts (Finger, 1997; Whitear 1992; Hansen et al., 2014) and mammals (Finger, 2003). Fibers innervating the papillae were shown to be AT-immunoreactive and some were also 5-HT-immunoreactive, which shows SCCs are actively signaling higher brain structures to induce a response.

The neurotransmitter in fish SCCs is unknown (Whitear, 1992). SV2 immunofluorescence was not present in SCCs, suggesting that synaptic vesicle transport is not used for neurotransmission of chemosensory information. Other types of vesicles are present in SCCs and are located in the sub-apical regions which are thought to be for apical membrane renewal (Whitear and Lane, 1983; Whitear 1992). Mammalian SCCs, which do have vesicles (Finger et al., 2003; Kinnamon, 2012) release acetylcholine to activate receptors on the trigeminal nerves to induce inflammation (Finger et al, 2003; Saunders et al., 2014). Lamprey and other teleosts SCCs do not possess the gene that encodes for gustducin (Ohmoto et al., 2011), this is the g-protein coupled receptor that mammalian SCCs use to activate phospholipase C (Saunders et al., 2014), and therefore lampreys may use a different g-protein cascade pathway to stimulate the IP₃ mediated pathway for SCC synapse activation. Although the full lamprey genome was not sequenced at the time Ohmoto et al. published their paper and further investigations could be performed. Fish SCCs also do not show any ecto-ATPase activity ruling out ATP as a neurotransmitter (Kirino et al., 2015), which is the neurotransmitter released by taste cells in mice (Finger, 2005). It has been suggested that chemosensory cells may use

amino acids as their neurotransmitter which would account for the absence of vesicles near the synapse (Roper 1989; Whitear, 1992). Further exploration of signaling and neurotransmission pathways of lamprey SCCs is needed to elucidate SCC activation and synapse signaling to the nervous system.

CONCLUSION

This study has shed light on SCCs in basal lineage vertebrates. In the sea lamprey, gill SCC abundance is greatest during the adult stage, when lampreys do not feed, but migrate to spawning grounds and select sites then spawn and die. These SCCs contain the enzyme phospholipase C, indicative of an IP₃ transduction cascade; the calcium binding protein calretinin and the biogenic amine 5-HT, but not the synaptic vesicle protein SV2. Nerve fibers reach the SCC basolateral region and serotonergic fibers remain in the lamina propria in the basal region of the papillae. These findings show homology between lamprey SCCs and the lamprey taste buds and to SCCs in derived vertebrates. This is the first study to show phospholipase C in the SCCs of non-mammalian vertebrates. Future studies can expand on these findings by determining SCC physiological and behavioural responses to stimulatory substances.

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TABLES

Table 2.1 List of antibodies used during immunohistochemical experiments

Name	Host	Source & catalog No.	Dilution	Lot	Immunogen
Calretinin	Rabbit	Swant, 7699/3, 7697	1:1000	1893- 0114	Recombinant human calretinin
Phospholipase C	Squid	Dr. Jane Mitchell, U of Toronto	1:1000		purified from the cytosol of squid photoreceptors
5-HT	Rabbit	Immunostar, 20080	1:3000	1131001	Serotoninformaldehyd e- BSA conjugate
a-tubulin	Mouse	Sigma, T 7451, clone 6-11B-1	1:1000	051M47 70 062M48 41V	Aceylated tubulin from the outer arm of Strongylocentrotus purpuratus
SV2	Mouse	DSHB, SV2 A	1:100	7/2/15	Purified synaptic vesicles

Table 2.2 Papillar SCC density in the gill pore of juvenile, preovulated, pre spermiated and spawning (spermiated and ovlultated) adult sea lampreys. An area of 45 μm x 45 μm =2025 μm^2 was analyzed from three papillae per individual lamprey. Spawning individuals had reduced weights compared to pre-spermiated/pre-ovulated stage individuals due to the depositing of milt and eggs at spawning sites during capture from nests.

Group	Body Weight (g)	Body Length (cm)	Papillar SCCs / 2025 μm^2	Group average SCCs / 2025 μm^2
Juvenile (males)	187.5	48.5	1, 3, 4	2
	85.3	39	1, 2, 5	
	72	33	0, 1, 1	
	79	38	1, 4, 5	
Prespermiated males	181	42.5	6, 10, 12	9.89
	231.9	50	7, 14, 16	
	253.8	54	5, 8, 12	
Preovulated Females	280.5	52	9, 14, 15	14.44
	248.3	50	11, 12, 12	
	193.2	48.5	12, 19, 26	
Spawning Males	171	39.5	10, 20, 23	17.44
	175.7	43	17, 18, 26	
	288	47.5	13, 15, 15	
Spawning Females	165.1	44	15, 16, 22	21.56
	182.5	43	18, 22, 30	
	156.6	43	19, 24, 28	

FIGURES

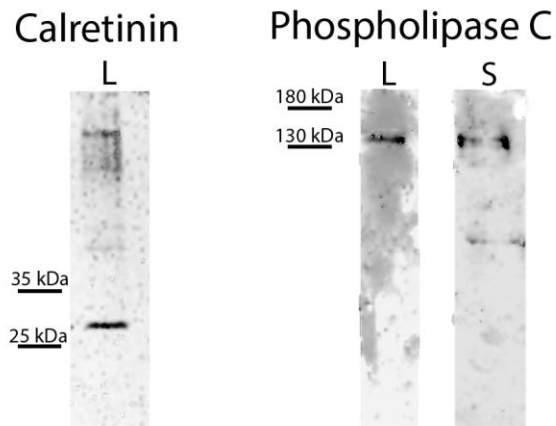


Figure 2.1. Western blotting of lamprey protein extracts with calretinin (CR) and phospholipase C 140 (PLC) antibodies.

Calretinin: L- lamprey brain extracts run on a 12% acrylamide gel. The antibody recognized a 29kDa calretinin protein.

Phospholipase C 140: L- lamprey gill papillae and S- Squid optic lobe tissue positive control (kind donation from Dr. Mary Baum and Dr. Dan Morse, University of California Santa Barbara) run on an 8% acrylamide gel. A band 140 kDa was recognized in both the squid and lamprey tissue extracts.

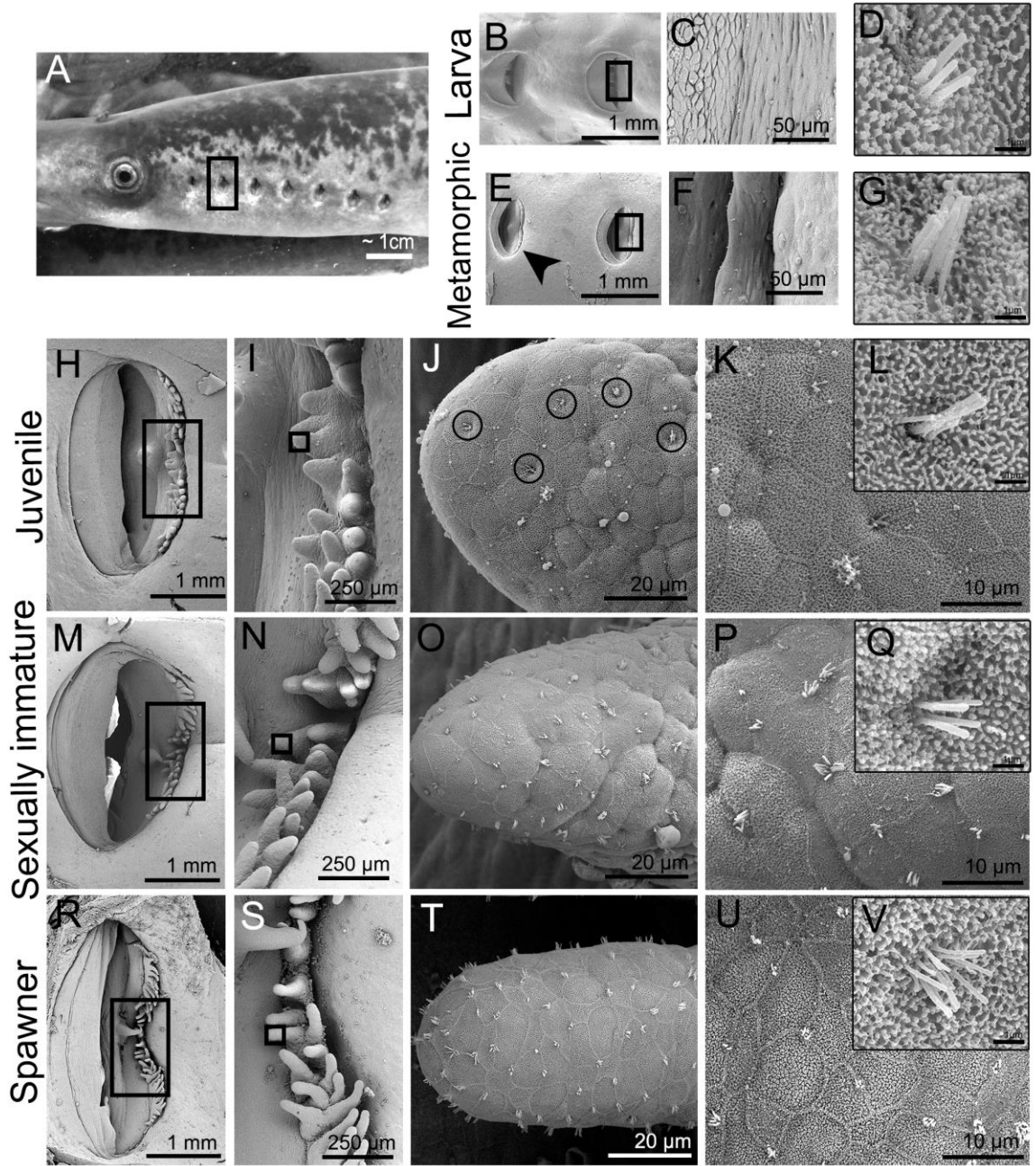


Figure 2.2 Gill pore SCCs during the sea lamprey life cycle. A. is taken under a stereomicroscope. B to W are scanning electron micrographs.

A: Spawning stage sea lamprey. There are seven brachial gill pores on the lateral surface behind the eye. The box shows the location of the second gill pore that was examined in the study.

B -D: Larval stage.

B. The caudal surface of the two gill pores is smooth. The area outlined by a box, shown at higher magnification in C shows the smooth caudal surface.

D. A single tuft of short microvilli was seen on the caudal surface of a larval gill pore.

E - G: metamorphic stage.

E. The caudal surface of the gill pores has a single vertical fold indicated by the black arrow. The area outlined by the rectangle is shown at higher power in F.

F. There is a low wavy contour on the surface on vertical fold on the caudal surface of gill pore.

G. An example of the sparse microvillar SCCs seen on the caudal surface of the gill pore.

H - L Juvenile stage

H. The caudal surface of gill pore has nub-like papillae. A central process is seen in the middle of the gill pore that possessed no SCCs during any life stages. The area enclosed by a rectangle is shown at higher power in I.

I. The abundant papillae have broad base and taper to a point. The area surrounded by the square is shown at higher power in J.

J. The papilla is covered by epidermal cells circled.

K. Microvilli are seen between epidermal cells.

L. A SCC on a papilla recognized by the microvillar tuft.

M - Q: pre-ovulated/pre-spermiated adult sexually immature stage

M. A row of papillae are located on the caudal surface of the gill pore. The area enclosed by a rectangle is shown at higher power in O.

N. Crowded papillae line the caudal surface of the gill pore. The area outlined by a square is shown at higher power in O.

O. Epidermal cells cover the papilla and tufts of microvillar are at the epithelial cell boundaries throughout the papilla.

P. The microvillar SCCs are abundant on the surface of the papilla.

Q. A typical SCC on the surface of the papilla.

R - V: spawner adult stage

R. There are abundant papillae on the caudal surface of the gill pore. The area enclosed by a rectangle is shown at higher power in S.

S. Abundant finger-like papillae populate the caudal surface of the gill pore. The area surrounded by the square is shown at high power in U.

T. Plentiful microvillar tufts are seen protruding from the surface of the papilla.

U. The epidermal cells are clearly demarcated and the microvillar SCCs between the epidermal cells.

V. The SCC cell contains abundant microvilli.

Scale bar is 1mm in B, E, H, M, R; 50 μm in C, F; 250 μm in I, N,S; 20 μm in J, O, T;

10 μm in K, P, U and 1 μm in D, G, L,Q, V.

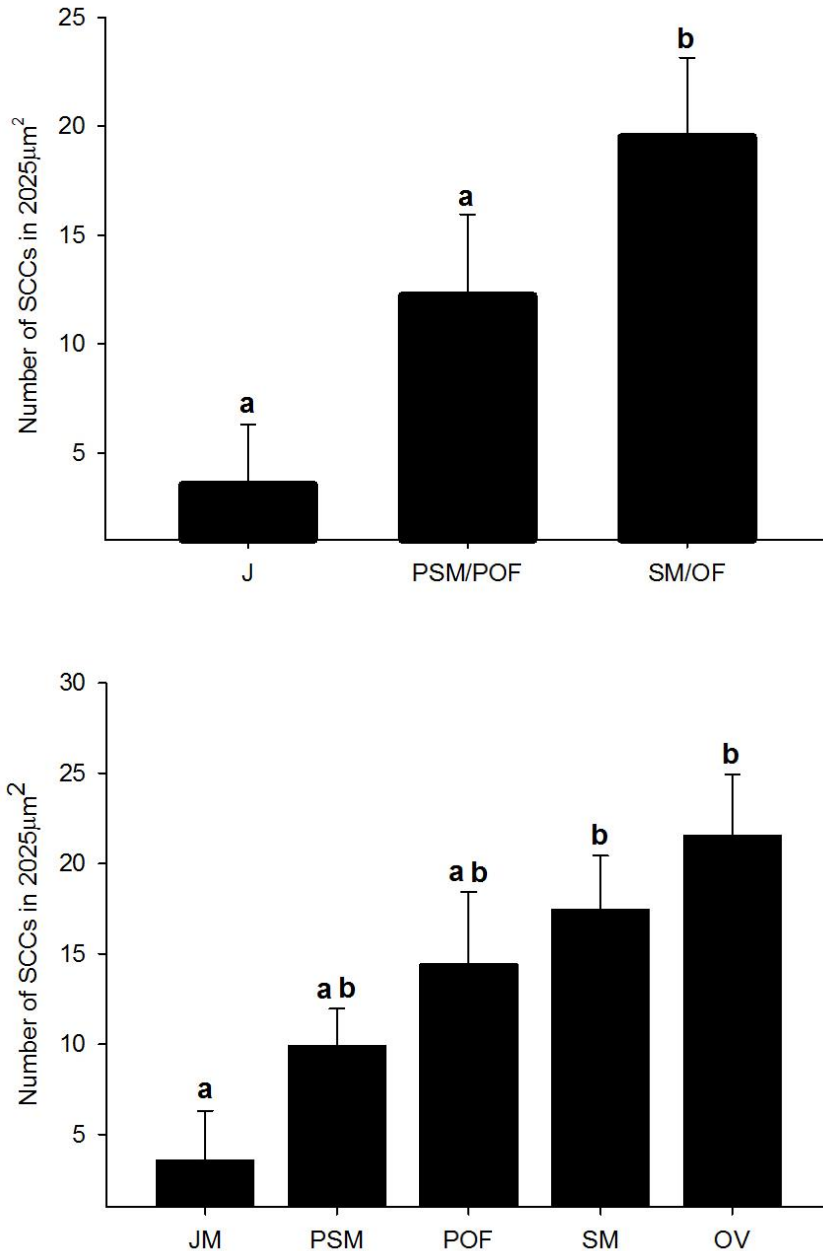


Figure 2.3 ANOVA comparison of SCCs observed on gill pore papillae in juvenile, sexually immature migrating adults and spawning adult sea lamprey.

A. Comparison of SCCs observed on gill pore papillae throughout the sea lamprey lifecycle, with sexes combined. One-way analysis of variance was performed on gill pore density data collected from juveniles (J; n = 3), combined sexes of sexually immature

adults (PSM/POF; n = 6) and combined sexes of spawning adults (SM/OF; n = 6), shown with standard error bars ($F = 20.689$, $p < 0.001$). Pairwise comparisons were made using *post hoc* Bonferroni-Holm test with statistical significance illustrated via groups a, b ($p \leq 0.001$).

B. Comparison of SCCs observed on gill pore papillae of males and females throughout the sea lamprey lifecycle. One-way analysis of variance was performed on gill pore density data collected from juveniles (J), pre-spermiated males (PSM), pre-ovulated females (POF), spermiated males (SM) and ovulated females (OF; n = 3 per group), shown with standard error bars ($F = 14.972$, $p < 0.001$). Pairwise comparisons were made using *post hoc* Bonferroni-corrected Tukey's test with statistical significance illustrated via groups a, b ($p \leq 0.001$).

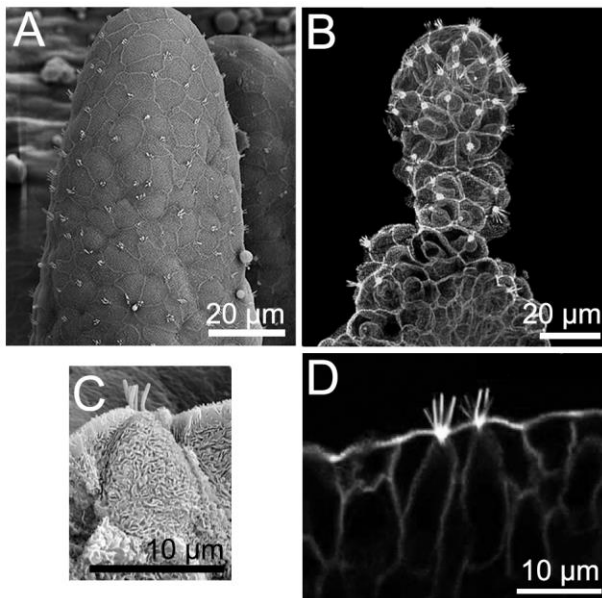


Figure 2.4. Under SEM and in sections SCCs on gill papillae are recognized by their microvillar surface. All are from spawning stage lampreys.

- A. A gill pore papilla visualized using SEM showing microvilli between epidermal cells.
- B. A confocal z-stack (15 slices, 1.32 μm z-step) of a sectioned papilla labeled with phalloidin showing the microvilli and actin adjacent to the cell membrane boundary of the papillar cells.
- C. Scanning electron microscopy of a fractured papilla revealing the shape of the SCC cell and microvilli protruding from the apical the surface.
- D. Phalloidin labeling of SCC microvilli and of actin adjacent to the cell membrane.

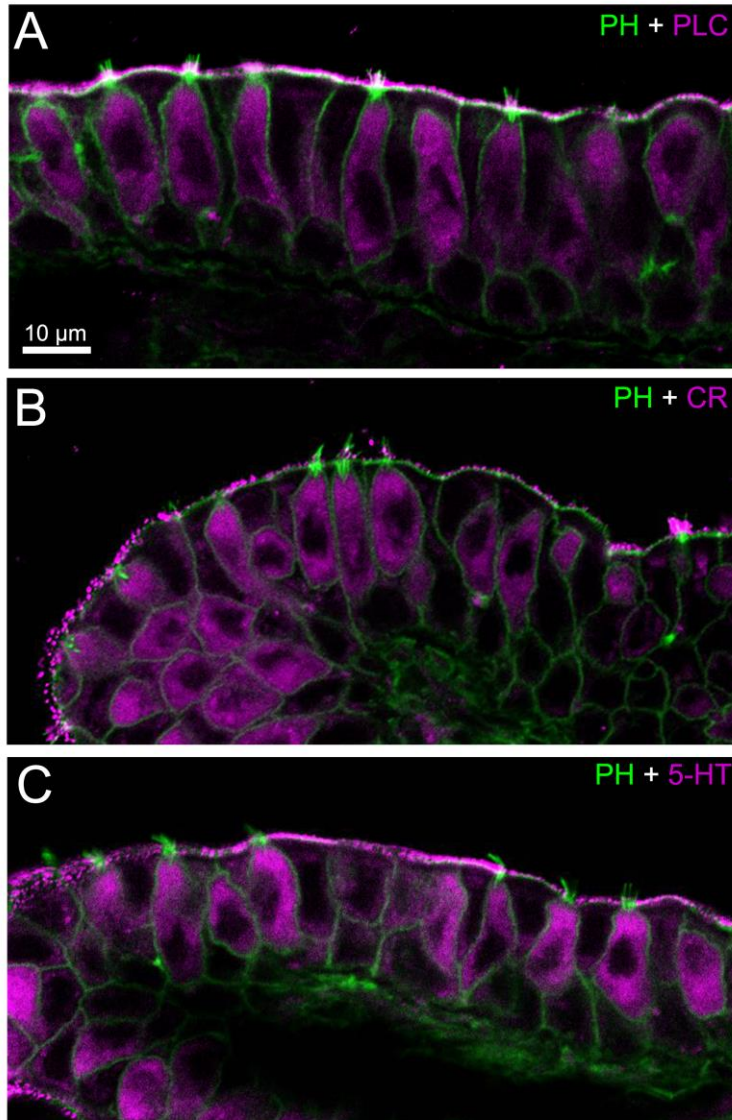


Figure 2.5. Double labeling of sea lamprey gill pore papillae. In all micrographs, phalloidin is green.

A. Phalloidin labels microvilli and the cell membrane boundary. Phospholipase C immunoreactivity (magenta) is cytoplasmic in the microvillous cells.

B. The cytoplasm is calretinin-immunoreactive for the cells covered by microvilli.

C. The microvillar cells are 5-HT-immunoreactive.

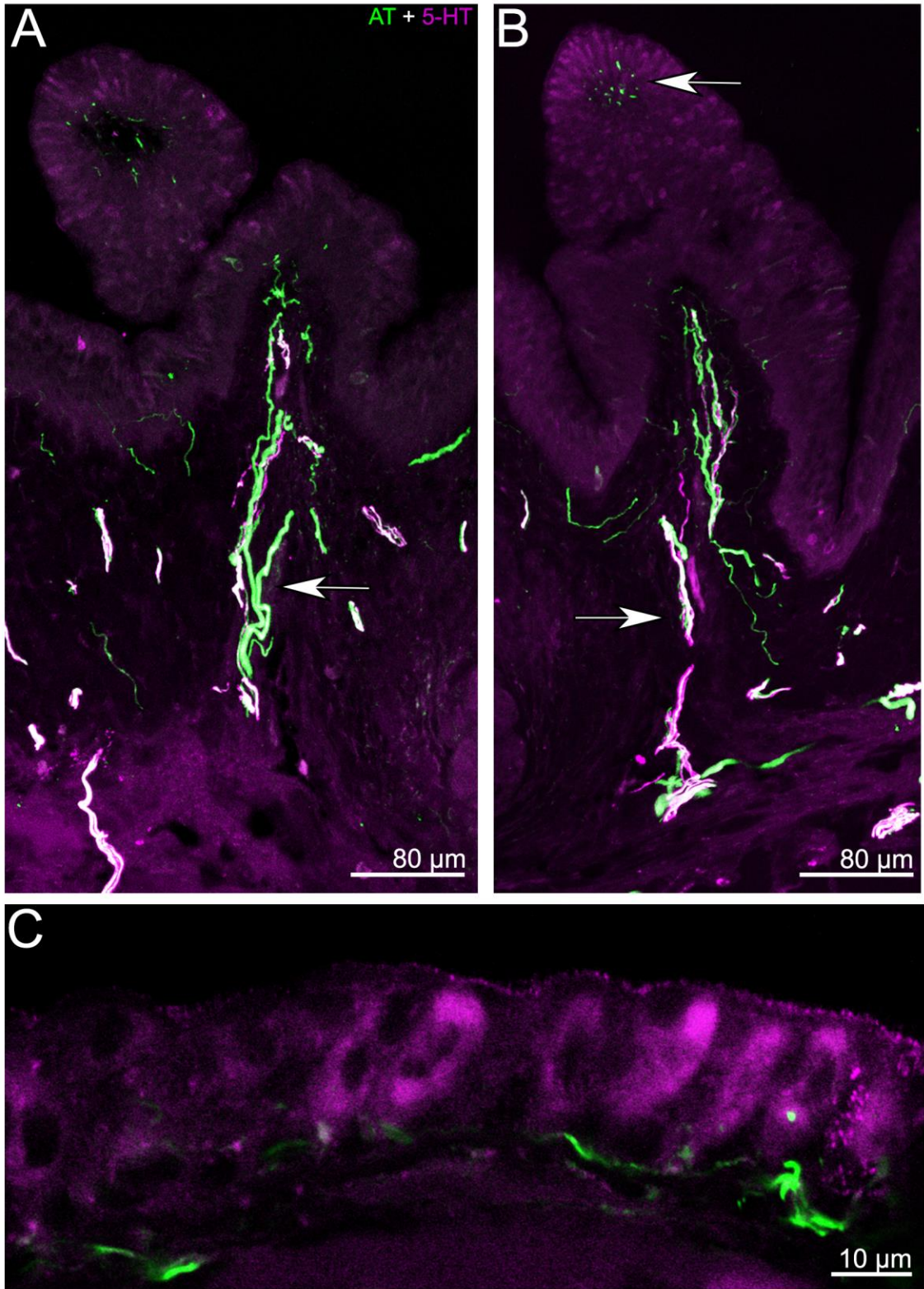


Figure 2.6. 5-HT and acetylated tubulin labeling in the gill papillae.

A and B. Confocal z-stack micrographs of acetylated tubulin-immunoreactive neuronal fibers projecting into the base of the papilla, and are also located in the lamina propria near the tip of the papilla indicated by the white arrows. Double labeling of some fibers with 5ht (magenta) and acetylated tubulin (green) is seen as white fibers. Acetylated tubulin fibers in green are more abundant and project all the way to the tip of a papilla compared to the 5-HT fibers in magenta that only project half way (top arrow in B). (A: 15 slices, 1.5 μm z-step, B: 16 slices, 1.5 μm z-step).

C. 5HT-immunoreactive cells also seen within the papillar epithelium approached by acetylated tubulin fibers (also seen in the top of B).

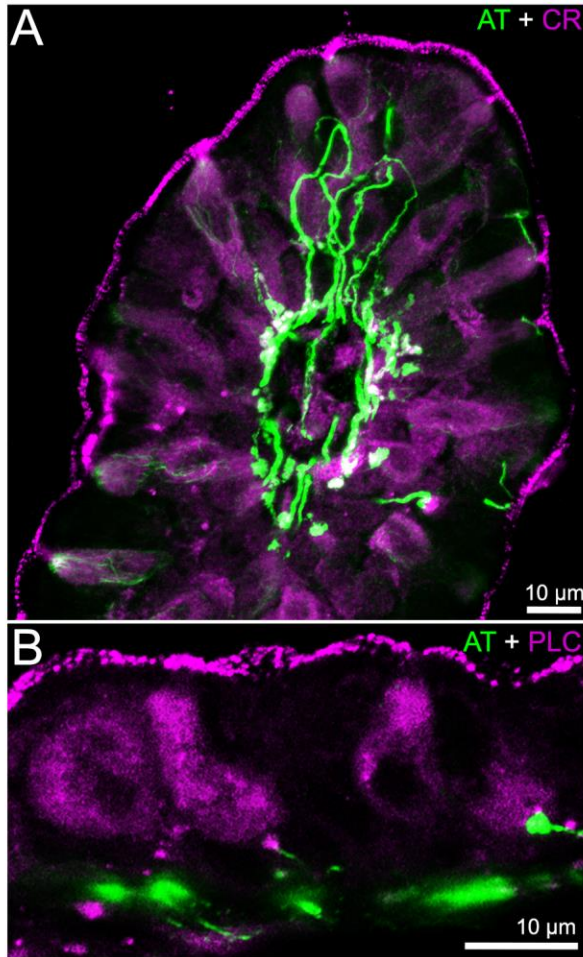


Figure 2.7. Acetylated tubulin fibers contacting the base of SCCs.

A. Confocal z-stack micrograph showing bright AT-ir fibers are shown projecting up the base of a papilla and branching outward toward the tip where they approach and encircle CR-immunoreactive SCCs. (10 slices, 0.73 µm z-step).

B. PLC-immunoreactive SCCs with an AT fiber running along their base.

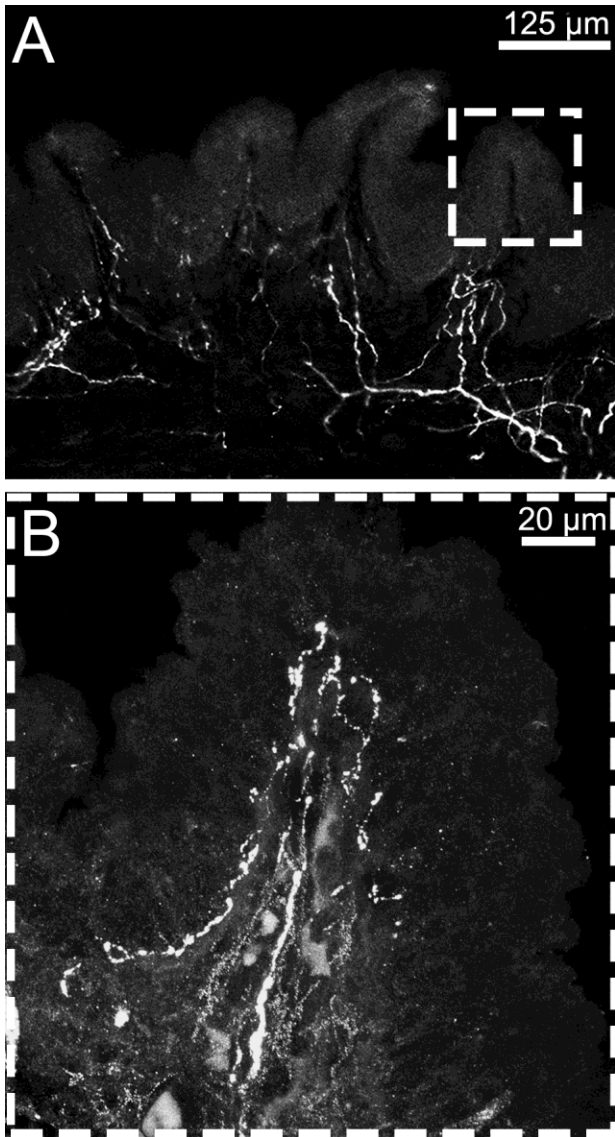


Figure 2.8. Anti- SV2 labelled nerve fibers extend into gill papillae.

A. Intensely labeled fibers are located deep into the connective tissue layer of the gill pore and extend into the papillae. The region enclosed by a square is shown at higher power in B.

B. Confocal z-stack showing SV2 labeled fibers project into the lamina propria of the gill papilla. (24 slices, 0.41 z-step).

**Chapter 3: DISTRIBUTION OF ORAL DISC AND DORSAL TAIL FIN
(OLIGOVILLOUS) SOLITARY CHEMOSENSORY CELLS DURING THE SEA
LAMPREY LIFECYCLE**

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CHAPTER 3 SUMMARY

The sea lamprey is an invasive species in the Great Lakes with a diffuse chemosensory system composed of finger-like papillae on the gill pores, oral disc and dorsal tail fins. Small microvilliar solitary chemosensory cells (SCCs) are located on these papillae and the function of this system is unknown. Life stages of the lamprey were examined for prevalence of SCCs on oral disc and tail papillae as a means for evaluating function as each life stage possesses phasic behaviours. Oral disc papillae were absent during the larval and metamorphic stages, but were present during the juvenile parasitic feeding stage, during the adult pre-ovulated/pre-spermiated migrating stage and during the adult spawning stage. Abundant SCCs were found during the late adult stages of life compared to earlier stages. This patterning was also found on the dorsal tail papillae with spawners possessing more SCCs, suggesting a function related to

end of life behaviours such as mating. Highly innervated tail papillae were revealed by labeling against acetylated tubulin. Various locations of SCCs along the body may also be collecting separate spatial chemosensory information as they are innervated by different cranial and spinal nerves and therefore may have different/specific functional purposes. This study aids in understanding the diffuse chemosensory system and how it varies based on the locations of SCCs along the surface of the body.

INTRODUCTION

Sea lampreys are an invasive species in the Great Lakes that use their oral disc to latch on to prey and suck their bodily fluids, with each individual capable of killing upwards of 40 lbs of fish in a 12-18 month feeding period (Applegate, 1950). They possess short finger-like tissue protrusions called papillae on the outer surface of the body that face the flow of water and collect sensory information (Whitcar and Lane, 1983; Baatrup and Doving, 1985). These papillae are present around the gill pore (See chapter 1), oral disc and on the dorsal tail fins (Whitcar and Lane, 1983B). Microvilliar solitary chemosensory cells (SCCs) are located on these papillae and make up the diffuse chemosensory system. The function of this diffuse chemosensory system is currently unknown.

The oral disc is composed of several rows of concentrically arranged teeth (Dawson, 1905) with a ring of mucus producing flat irregular leaf-shaped fimbriae that help the fish to create a secure suction on its prey, and an outer ring of finger-like papillae that collect sensory information (Cook et al., 1990; Khidir and Renaud, 2003). Electrophysiology recordings have shown these oral disc SCCs have a different chemosensory spectrum than that of the taste system as they respond to acetic acid, sialic acid, fish body

mucus, and trout water washings, over canonical taste solutions and amino acids (Baatrup and Doving, 1985A, B). Oral disc SCCs are innervated by the trigeminal nerve (Daghfous, 2015).

Lamprey dorsal fin SCCs have not been extensively studied. It is known that they occur on papillae on the posterior border of the fin in lampreys (Whitear and Lane, 1983), but the nerves that innervate these SCCs are unknown. Most of the research on fin SCCs has been done in rocklings (Teleostei, Gadidae: *Ciliata mustela*) where SCCs are present on their long undulating dorsal tail fin which is innervated by the recurrent facial nerve, (Kotrschal et al., 1993, 1998; Kotrschal and Whitear, 1988; Peters and Kotrschal, 1987) and in sea robins (Teleostei, Triglidae: *Prionotus carolinus*) SCCs are located on their free pectoral-fin rays and are innervated by spinal nerves (Bardach and Case, 1965; Finger, 1982; Kotrschal, 1995, 1996). Benthic rocklings will probe the water with their undulating dorsal fin to sample for mucous, indicating the presence of prey and predators (Kotrschal, 1995, 1996). Sea robin SCCs have been shown to respond to amino acids and extracts from marine invertebrates (Bardach and Case, 1965; Silver and Finger, 1984), indicating chemosensory-foraging related behaviours. Due to the variety of SCCs locations within fish species, and their varying chemical responsiveness and innervation patterns, it is likely the behavioural use of this diffuse chemosensory system is diverse (Finger 1997).

The objectives of the present study were to compare papillae and SCCs from the oral disc and dorsal tail fin locations using scanning electron microscopy to assess distribution and immunohistochemistry to assess innervation patterns across the sea lamprey lifecycle, as it has distinctive stages where specific behaviours occur such as

feeding, migration, or spawning. Comparing locations of SCCs may infer a function of the diffuse chemosensory system based on body positions highlighting a relationship between form and function.

MATERIALS AND METHODS

Experimental animals and tissue preparation

Sea lamprey, *Petromyzon marinus* larvae (n = 2), metamorphic stage (n = 2), juveniles (n = 4), pre-ovulated/pre-spermiated migrating adult stage (n= 24) and spawning adult stage fish (n = 30) were caught from Lake Huron and the surrounding tributaries by Hammond Bay Research Station, MI and transported to the University of Windsor Biology Building. Each animal's weight and length were measured and recorded (*See Appendix B, Table 1). The lamprey were euthanized by anaesthetic overdose (1 g/L MS-222), and dissected to collect their oral disc and anterior and posterior dorsal tail fins. All protocols were approved by the Canadian Council for Animal Care. Tissue collected from the right side of the oral disc was drop fixed for scanning electron microscopy in 5% glutaraldehyde 0.2M sodium cacodylate fixative and tissue collected from the left side was drop fixed in 4% paraformaldehyde (PFA) fixative for less than 24 hours for immunohistochemistry (*See Appendix A, protocols and solutions). Tail samples from individuals were either drop fixed in glutaraldehyde for SEM or into PFA for immunohistochemistry.

Tissue preparation

Oral discs and tail clippings were removed from fixative and washed with either 0.1M sodium cacodylate wash buffer for SEM or 0.1M PB for immunohistochemistry

before being examined under a dissection microscope. Oral disc samples were orientated so papillae could be examined (Fig. 5B) and a tissue sample was taken from the ventral-medial portion of the disc. Both the anterior and posterior fins were studied but only the posterior tail data is presented here as there were more abundant papillae and a larger surface area to examine (Whitewar and Lane, 1983B). Posterior tail samples were cut from the most posterior portion of the fin clip where papillae were identified (Fig. 5C). These tissue samples were prepared according to the materials and methods listed in chapter 2.

(See Chapter 2, materials and methods, "*Tissue preparation for SEM*", "*Tissue preparation for sectioning*", and "*Immunohistochemistry*").

RESULTS

Oral disc papillae and SCCs during the lamprey lifecycle

Oral discs were examined from larval to spawning stage lamprey. The right side of each oral disc was examined and a sample from the bottom portion of the disc was analyzed under SEM. The larval stage did not possess an oral disc with fimbriae or papillae (Fig 1.A). A smooth oral hood surface was observed around an opening of prominent oral cirri. Metamorphic stage lamprey did not have papillae (Fig 1B, 2A) but did have a ring of fimbriae around their oral disc with well-developed teeth (Fig. 1 B). In the juvenile stage, papillae were located outside the ring of fimbriae. In juvenile parasitic lamprey papillae were slightly smaller than their fimbriae (Fig. 1C). In the pre-ovulated/pre-spermiated adult stage, the papillae and the fimbriae were large (Fig. 1D). Lastly the largest papillae appeared to be located in the spawning adult stage compared to the other age classes examined (Fig. 1E).

In juvenile lamprey, surface of the oral disc papillae was smooth (Fig. 2B), with very few SCCs found (Fig. 2C). The papillae on pre-ovulated/pre-spermiated adult lampreys had some mucus build up which fixed to the tissue and created a rough surface at the tip of examined papillae (Fig. 2E) but did have little dips where SCCs were located. Figure 2F shows a zoomed in view of the pre-ovulated/pre-spermiated adult papilla in 2E, several SCCs were evenly distributed along the surface. Examples of the SCCs are shown in Figure 2 G and H. The spawning adult stage has large papillae, with abundant SCCs scattered all over the epithelial surface (Fig. 2I). Several large SCCs were identified (Fig. 2J), with several microvilli per SCC (Fig. 2K and L). Comparison of the SCCs across the three life stages shows the same general characteristics that match Whitear and Lane's (1983B) description of oligovillous cells possessing several independent microvilli protruding from the surface (Fig. 2 D, G, H, K, L). Over all, SCC abundance seemed to increase with papillae and animal size and life stage, as the spawning adult stage had the most SCCs observed and the largest oral disc papillae.

Dorsal tail fin papillae and SCCs during the lamprey lifecycle

Tail clippings were examined from larval, metamorphic, juvenile, pre-ovulated/pre-spermiated adult stage and spawning adult stage lamprey. Fin clips from the posterior tail were taken from the most posterior portion for each individual as that area possessed the most papillae (Whitear and Lane, 1983). The larval stage tail fin had a very smooth surface with no papillae (Fig. 3A), with no SCCs observed. The metamorphic stage also had a smooth surface with small indentations that look like little nubs but not yet papillae and no SCCs found (Fig. 3B). The juvenile stage had small papillae that were square shaped with a pointed tip, roughly 50 μm in height and very few SCCs identified

(Fig. 3C). The papillae of pre-ovulated/pre-spermiated adults were slightly larger than in the juvenile stage, approximately 80-100 μm in height, they were taller than they were wide (Fig. 3D). The spawner adult stage had the largest papillae of the stages examined, over 100 μm wide (Fig. 3E.) Overall, papillae seemed to increase in size correlated to body size/developmental stage.

Comparison of the juvenile stage to the spawning adult stage shows how SCC distribution changes from a few SCCs during the juvenile stage (Fig. 4A) to many SCCs during the spawning adult stage (Fig 4E). The SCCs also change from being monovillous or possibly coalesced during the juvenile stage (Fig. 4 B, C, D), to having several well-defined microvilli per SCC (Fig. 4 F, G, H, I).

Innervation of spawner oral disc and tail papillae

Oral disc and tail tissues were examined under a stereoscope to identify papillae then sectioned and labeled using immunohistochemistry for acetylated tubulin (Fig. 5 D and E) and 5-HT (Fig. F and G). Oral disc papillae were hard to recognize in cross-sections due to the abundance of fimbriae (Fig. 5B), but were positively identified by their triangular shape compared to the irregular leaf-shaped fimbriae (Fig. 5D). Small acetylated tubulin-immunoreactive fibers were in the lamina propria of the papillae. Tail papillae (Fig. 5C) were sectioned in cross and longitudinal sections. Bright acetylated tubulin-immunoreactive fibers can be seen in the lamina propria, branching towards the tip of each papilla (Fig. 5E). 5-HT immunoreactive fibers were not seen in the oral disc papilla, but were seen in tail papillae (Fig. 5F, G). These fibers were present in the lamina propria, from the base to the apex of the tail papilla.

DISCUSSION

The results of the present study show that spawning adult stage lamprey possessed large papillae with many SCCs on their oral disc and tail papillae compared to the few observed SCCs from pre-ovulated/pre-spermiated adults or from the juvenile stage. Larval and metamorphic stages did not possess any oral disc or tail papillae. In general, oral disc papillae appear to have slightly less observed amounts of SCCs than tail papillae (and gill papillae, *see chapter 1). Whitear and Lane in 1983 also reported finding more SCCs on the gill pore and dorsal fins of *Lampetre planeri* than on the oral disc papillae.

While oral disc papillae do not appear to have as many SCCS as in the other body locations, they still may play an important role in collecting sensory information. It is thought that oral disc papillae aid lamprey in finding a good attachment site as the disc is not flared out while swimming and therefore the outer papillae would be the first structures to come into contact with potential prey (Cook et al., 1990). Recordings from adult brook lamprey oral disc papillae have shown they respond to dead trout water and sialic acid found in body mucus (Baatrup and Doving, 1985b). In taxonomic comparisons of adult lamprey species, blood feeding parasitic lamprey have been shown to possess more oral disc papillae than flesh eating and non-parasitic lamprey (Khidir and Renaud, 2003). Together, these findings suggest that oral disc papillae may function during an initial response in prey detection during the parasitic stages.

As we have found that SCCs increase in abundance during the spawning adult stages, the function may change to aid in nest building and spawning behaviours. Male spawning lampreys migrate to streams ahead of females and will collect rocks to construct nests in riffle areas (Applegate, 1950; Manion and Mclain, 1971). Oral discs are

used to pick up rocks (Applegate, 1950, Johnson et al., 2015) and collecting sensory information at this stage may be beneficial for nest building. During spawning, males and females intertwine and males attach their oral disc on the back of females heads (Johnson et al., 2015), perhaps oral disc papillae, as well as genital papillae with SCCs (Whitear and Lane, 1983), collect sensory information about mate choice during spawning.

The function of the diffuse chemosensory system may be diverse in many species, but at a basic level in aquatic species, it seems to be for scanning ambient water for stimuli as SCC locations face the flow of water over the surface of the body (Kotrschal, 1995). Benthic rocklings will probe the water with their undulating dorsal fin to sample the presences of other fish (Kotrschal, 1995). This behaviour allows the fish to remain motionless in a shelter while remaining able to predict the presence of a fish upstream. While beneficial, this behaviour also disrupts stimuli concentrations as the flow of water is affected and means that the fish would need to interpret fin frequency and flow velocity to determine the change in stimuli detection (Kotrschal, 1995, 1996). Lamprey dorsal fin SCCs cannot independently move, as in the rockling, and therefore stimuli detection is likely produced by active swimming to sample the environment and maximize the collection of spatial information (Kotrschal, 1996). This hypothesis is further supported by odour-mediated locomotor responses, where odour cues will stimulate movement (Derjean, 2010) and this pathway could also be utilized by SCCs where olfactory stimuli could be combined with SCC information and initiate movement (Kotrschal, 1995). Testing of the olfactory versus the diffuse chemosensory system's input on influencing behaviour may be challenging to definitively conclude, as stimulatory substances for both systems are applied directly in the water and that

olfactory-impaired fish will not behave normally (Essler and Kortschal, 1994) and therefore it would be difficult to infer if behaviours are due to the diffuse chemosensory system or an artifacts of experimentation.

Nerve fibers labelled using acetylated tubulin in the papillae show that these SCCs are capable of sending their signals to higher brain structures. Innervation of the oral disc by the trigeminal nerve (Daghfous, 2014, 2015) and the tail possibly by the spinal or cranial nerves (Finger, 1997) may indicate that each is involved in different functions and behaviours as the nerves connect to different parts of the brain. More research is needed on the chemosensory spectrums of each location and how receptors detect stimuli from the environment.

CONCLUSION

Studying the SCCs of a basal lineage vertebrate will help to shed light on the function and development of the diffuse chemosensory system. Oral disc and tail papillae were largest during the spawning adult stage and possessed abundant SCCs, indicating that the diffuse chemosensory system may be used during migration, nest building and reproductive behaviours. Comparison of SCCs across body locations as well as to other fish species with specialized appendages and various life history strategies contrasts and highlights an evolutionary relationship between form and function of this diffuse chemosensory system. As Kurt Kortschal said in his 1995 paper “if we do not understand functions and biological roles of SCCs, it will not be possible to explain behaviour and ecology of fishes”.

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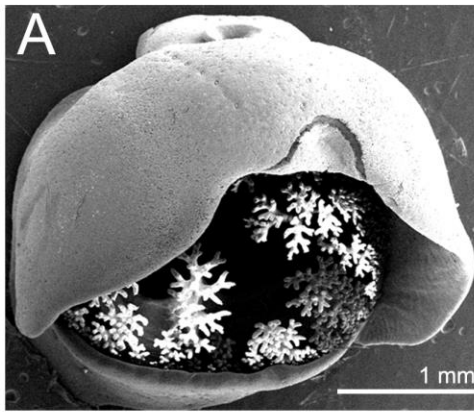
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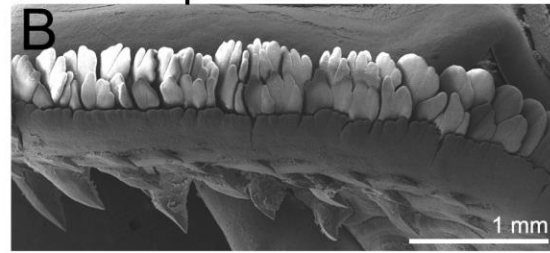
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FIGURES

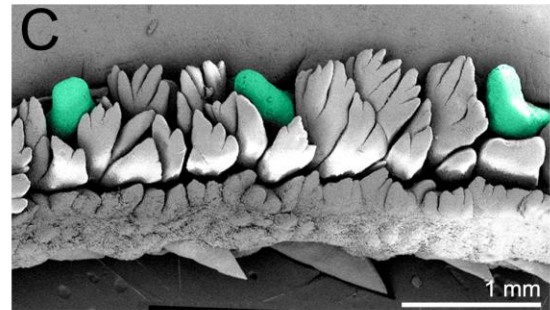
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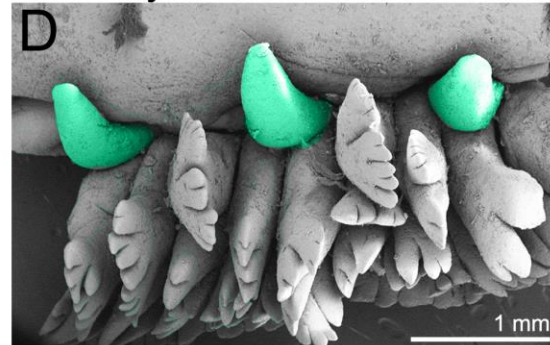
Metamorphic



Juvenile



Sexually immature



Spawner

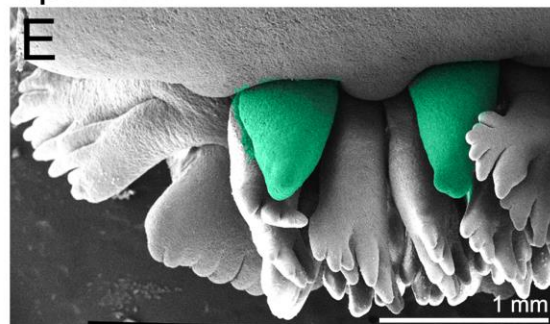
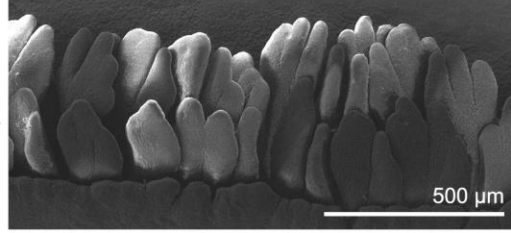


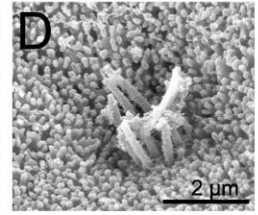
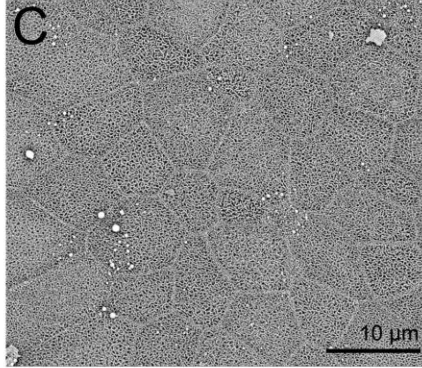
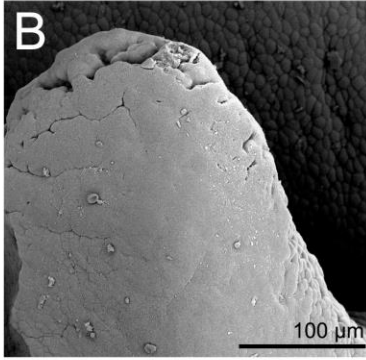
Figure 3.1. Oral disc papillae comparison by scanning electron microscopy during larval, metamorphic, juvenile, pre-ovulated/pre-spermiated adult stage and spawning adult stage lamprey, with green pseudo-coloured papillae.

- A. Larval oral cavity. A smooth edge can be seen all the way around the oral cavity. Large filtering tissue protrusions (oral cirri or tentacles) can be seen inside the mouth.
- B. Metamorphic oral disc. Only fimbriae are present around the periphery of the oral disc at this stage. Teeth are present at this stage, seen in the lower portion of the micrograph.
- C. Juvenile oral disc. Three papillae in green on the periphery of the oral disc can be seen poking up in between large fimbriae.
- D. Sexually immature (pre-ovulated/pre-spermiated) adult oral disc. Three papillae in green close in size to the fimbriae are shown.
- E. Spawner adult oral disc. Two large papillae in green can be seen outside the fimbriae lining the oral disc.

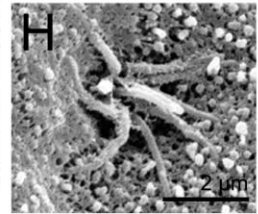
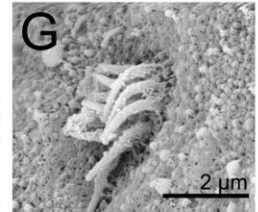
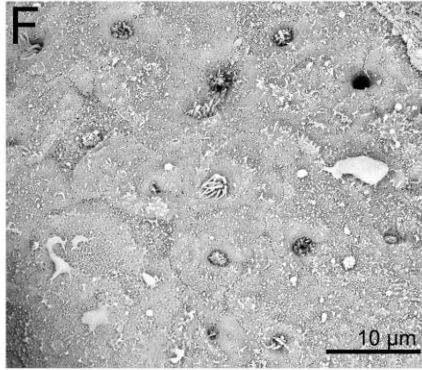
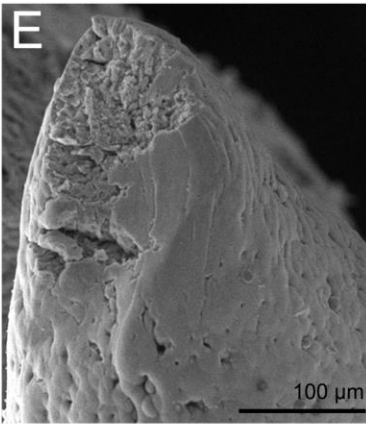
Metamorphic



Juvenile



Sexually immature



Spawner

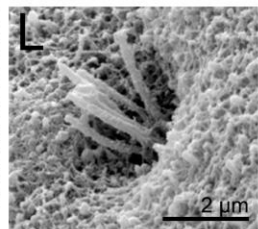
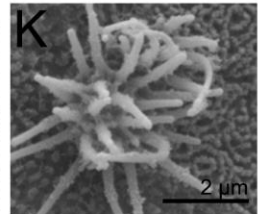
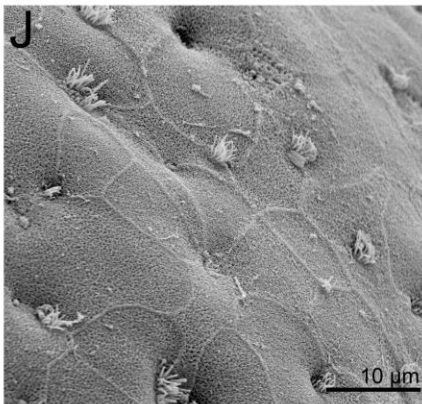
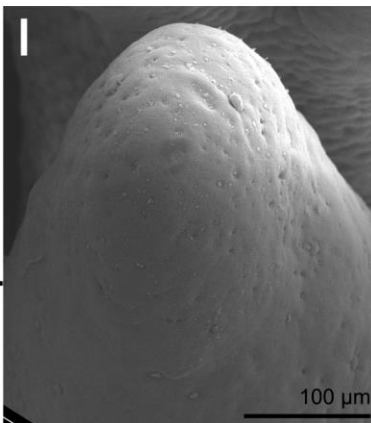


Figure 3.2. Oral disc papillae and SCC distribution by scanning electron microscopy among juvenile, pre-ovulated/pre-spermiated adult stage, and spawning adult stage lamprey.

A. Metamorphic oral disc. No papillae are present at this stage, only fimbriae can be seen.

B-D: Juvenile

B. Juvenile papillae with a smooth surface

C. The ridges of epidermal cells can be seen creating a smooth surface.

D. An example micrograph of an SCC taken from a juvenile oral disc papilla.

E-H: Sexually immature (pre-ovulated/pre-spermiated) adult stage

E. A smooth papilla is shown with little concave dips where SCCs are present.

F. Several SCCs can be seen in the field of view. Mucus present creates a rough epidermal surface.

G and H: SCCs from F under high power magnification. Several microvilli can be seen protruding in all directions.

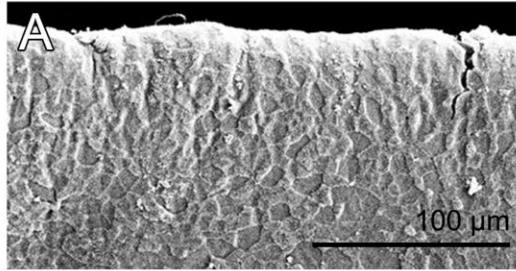
I-L: Spawner adult stage

I. A large spawner oral disc papilla is shown, speckled with SCCs.

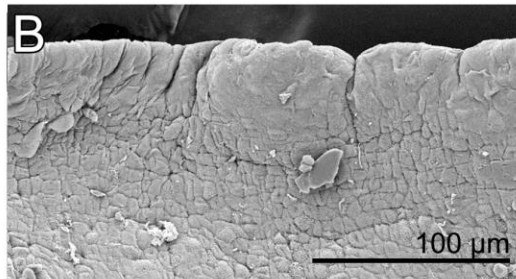
J. Several large SCCs can be seen in the field of view in between epidermal cells.

K and L: SCCs from J under high power magnification.

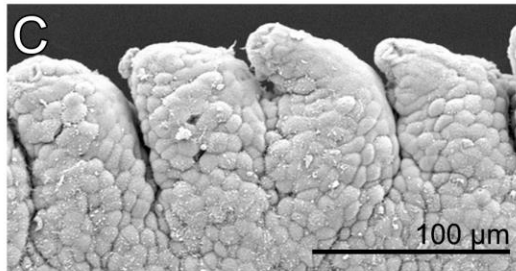
Larva



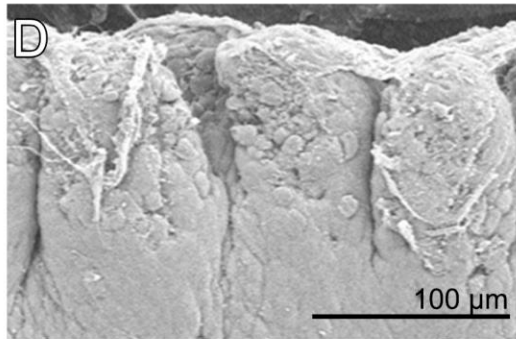
Metamorphic



Juvenile



Pre-ovulated/
Pre-spermiated



Spawner

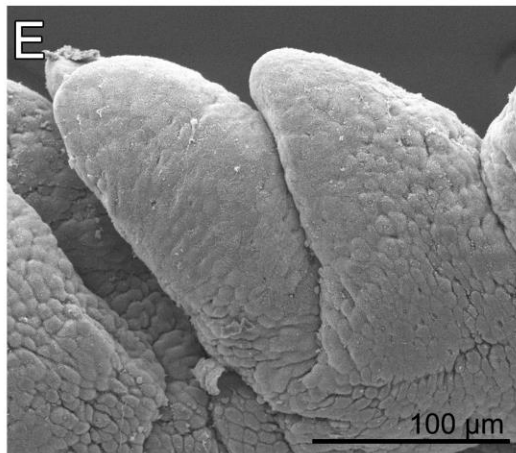
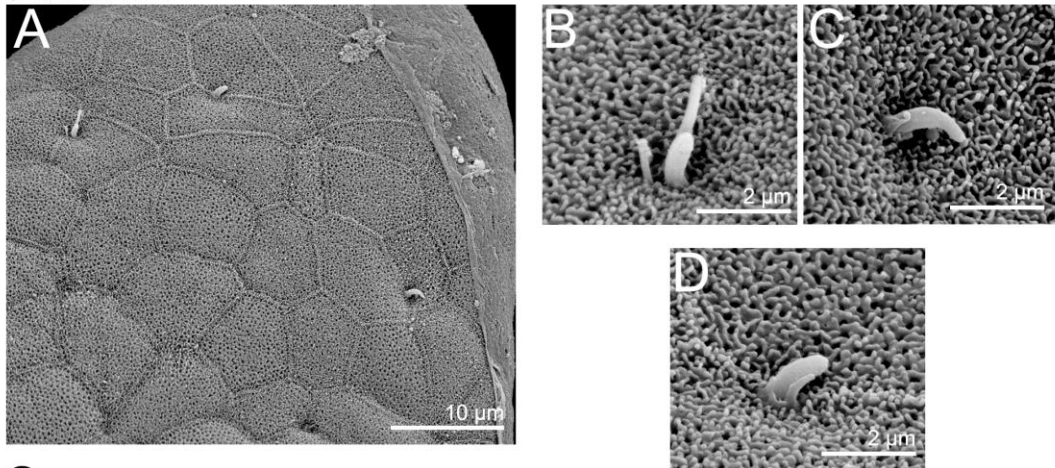


Figure 3.3. Tail papillae comparison by scanning electron microscopy from larval, metamorphic, juvenile, pre-ovulated/pre-spermiated and spawning stage lamprey.

- A. Larval tail clipping. The top of the tail clipping is a smooth surface with no papillae.
- B. Metamorphic tail. Small indentations that create nub-like papillae can be seen on the top of the tail.
- C. Juvenile tail. Small papillae are present at this stage.
- D. Pre-ovulated/pre-spermiated adult tail. Large papillae are present, with mucus residues on the tips.
- E. Spawning adult stage papillae. Large papillae are abundant at this stage.

Juvenile



Spawner

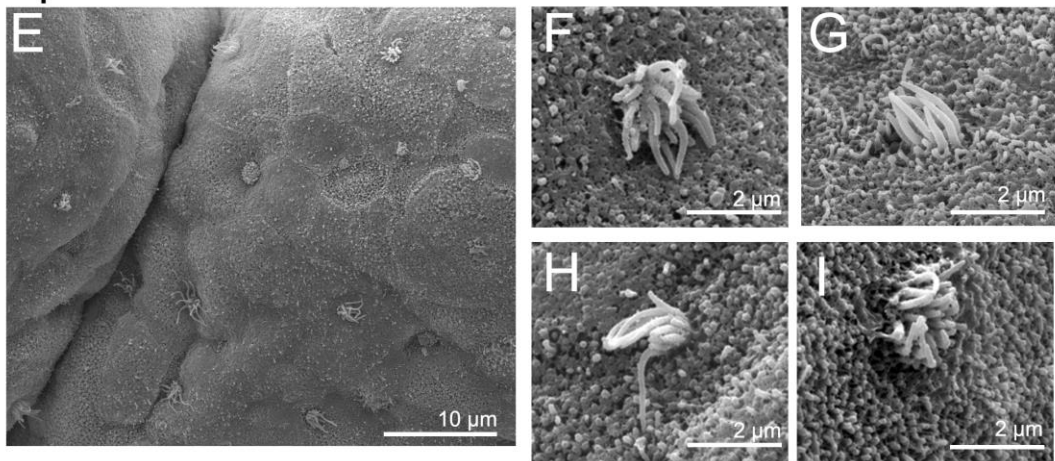


Figure 3.4. Scanning electron microscopy of tail papillae and SCCs in juvenile and spawner lamprey.

A-D: Juvenile

A. Few microvillar tufts are seen in between prominent epidermal cells.

B-D. Examples of SCCs. There are few microvilli per SCC and the microvilli seem to be coalesced.

E-I: Spawner

E. Many SCCs are located across the surface of epidermal cells.

F-I. Several microvilli per SCC can be seen. They range from long protrusions to short stubs.

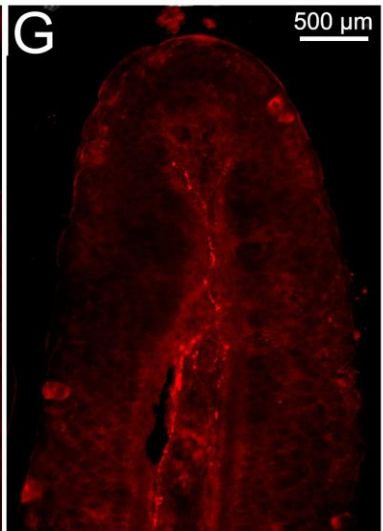
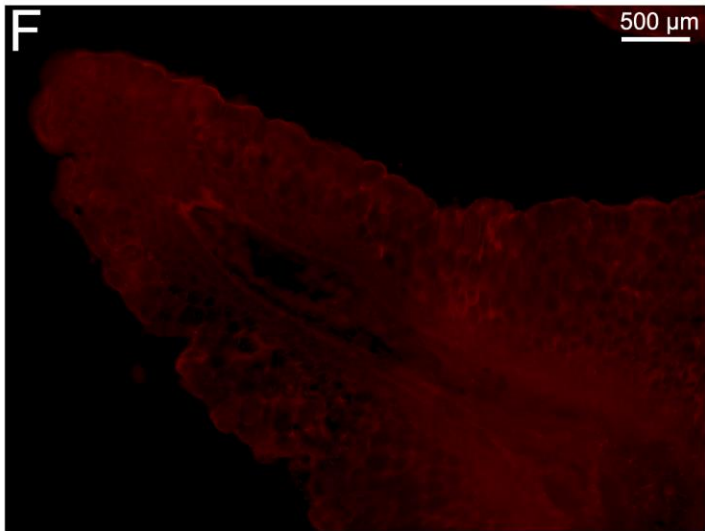
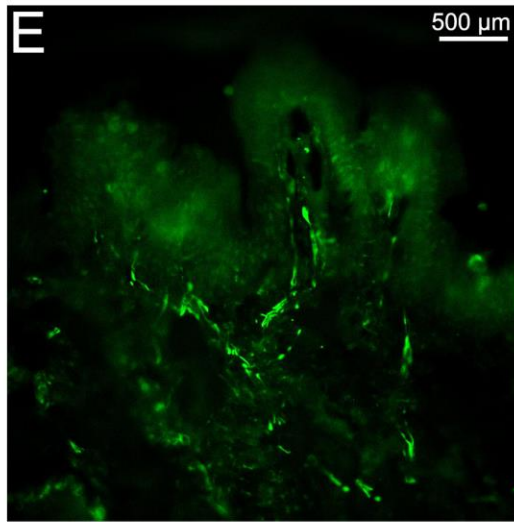
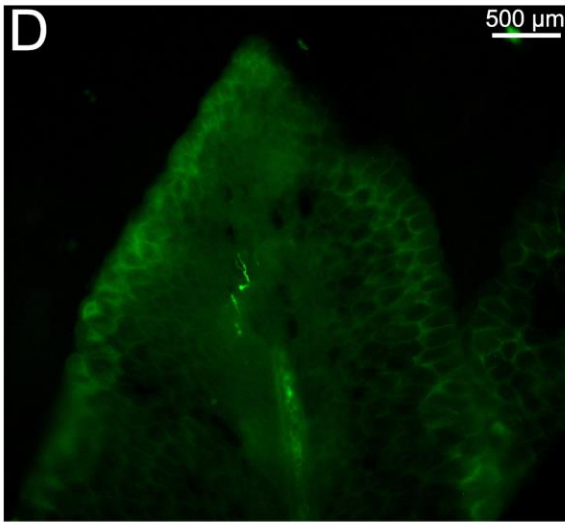
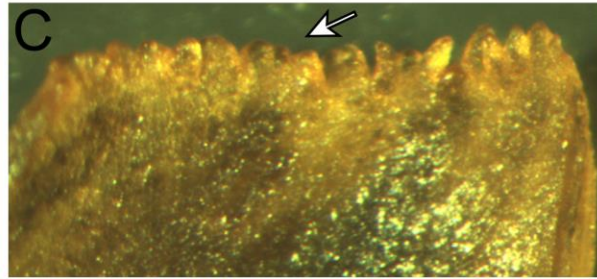
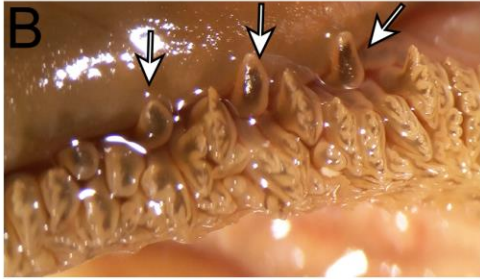


Figure 3.5. Labeling of nerve fibers in oral disc and tail papillae using acetylated tubulin and 5-HT antibodies. B and C are stereomicroscope images. D to E are epifluorescence

- F. Drawing of a spawner lamprey showing the location of the oral disc papillae and tail papillae.
- G. Stereoscope micrograph of a spawner oral disc. Three large papillae (indicated by the arrows) are outside a ring of fimbriae.
- H. Stereoscope micrograph of a spawner posterior tail fin. Papillae (indicated by the arrow) are ridge-like along the dorsal surface.
- I. Epifluorescence micrograph of an oral disc papilla labeled with acetylated tubulin. A nerve fiber is located in the lamina propria.
- J. Epifluorescence micrograph of tail papillae labeled with acetylated tubulin. Many bright fibers are located near the base and the tip of each papilla.
- K. Epifluorescence micrograph of an oral disc papilla labeled with 5-HT. No labeling is present in this papilla.
- L. Epifluorescence micrograph of a tail papilla labeled with 5-HT. A bright fiber runs is located in the lamina propria of the papilla.

Chapter 4: THESIS SUMMARY

The objectives of this thesis were to study the distribution, neuroanatomy and biochemical characteristics of the sea lamprey solitary chemosensory cells that make up the diffuse chemosensory system in the hopes of understanding the function of lamprey SCCs, their involvement in life history behaviours, and to compare aspects to SCCs in derived vertebrates.

In the chapter 2, gill pore SCCs were examined across the life cycle of the sea lamprey using SEM and immunohistochemistry techniques. Spawning adult stage individuals possessed abundant SCCs compared to that of larval, metamorphic, juvenile, and pre-ovulated/pre-spermiated adult stage lampreys. Since adults (specifically spawning stage individuals) possessed more SCCs than younger age classes, it is postulated that the diffuse chemosensory system may be used for behaviours that occur later in life, such as migrating and spawning, as these adults are not feeding and are investing valuable energy into developing this chemosensory system. Prominent calretinin, 5-HT and phospholipase C labeling was present within SCCs double labeled with phalloidin and acetylated tubulin-immunoreactive fibers approached the base of these cells. This was the first report of phospholipase C labeling within non-mammalian SCCs and indicated that lamprey SCCs may use a conserved IP₃ mediated transduction cascade like mammalian SCCs (Saunders et al., 2014) and olfactory sensory neurons (Hansen et al., 2003). Calretinin and 5-HT patterning showed homology to the lamprey taste system and to that of other vertebrate SCCs (Barrerio-inglesias et al., 2008C, Hansen et al., 2014; Saunders et al., 2014).

In chapter 3, oral disc and tail papillae were examined across age classes to understand how the diffuse chemosensory system varies based on the locations of SCCs along the surface of the body. Papillae were present in juvenile, pre-ovulated/pre-spermiated adult stage and spawning adult stage lamprey but were absent during larval and metamorphic stages. Similar to chapter 2 studying the gill pores, adult lamprey possessed abundant SCCs on these papillae compared to younger stages. In the adults, these papillae were also shown to be innervated. Based on the distribution of SCCs being most abundant during the spawning adult stage and based on behaviours such as oral disc latching on to rocks during nest building and during spawning and tail movements possibly for water sampling (like the SCCs of the rockling's undulating dorsal tail fin), it is postulated that locations of SCCs on papillae in the mouth, gills and tail may serve various purposes for migratory and reproductive behaviours by collecting and relaying separate spatial chemosensory information to specific cranial and spinal nerves that project to different areas in the brain.

The sea lamprey, a basal lineage vertebrate, represents a phylogenetically ancient component of this diffuse chemosensory system which still exists in modified forms in higher vertebrates. In rocklings, SCCs are located on their dorsal fin and function for prey/predator detection (Kotrschal et al., 1993, 1998; Peters and Kotrschal, 1987), and in sea robins it functions during foraging behaviours (Bardach and Case, 1965; Kotrschal, 1995), in mammals (rodents) airway SCCs function to initiate a protective reflex against bitter irritants and toxins (Finger et al., 2003, Saunders et al., 2014). SCCs have also been shown in alligator nasal cavities (Hansen, 2007), and cow laryngeal taste buds (Tizzano

et al., 2006). All these locations seem to be for collecting chemosensory stimuli important for influencing behaviour and survival.

The sea lamprey has been shown to have olfactory-mediated locomotor responses from stimulation by odours and pheromones in the water (Derjean et al., 2010; Daghfous, 2012). This sensory-locomotor process has been exploited against the lamprey by applying male released pheromones to lure females into traps for population control management (Johnson et al., 2009). A juvenile parasitic sea lamprey can eat about 40 lbs of fish in their short feeding period (Applegate, 1950), which has a large effect on the fishing industry in the Laurentian Great Lakes. The mechanisms underlying the diffuse chemosensory systems responses to stimuli and whether it also induces a locomotor response are still poorly understood and more research is needed. This knowledge could then be used for stronger population control methods by making more effective traps that function by affecting multiple chemosensory systems that would benefit not only the ecosystem but also the economy.

This study is the first to look at SCCs across the lamprey lifecycle and to show localization of phospholipase C to non-mammalian SCCs (mammalian SCCs: Saunders et al., 2014), indicating that lamprey SCCs may also utilize an IP₃ mediated transduction cascade. Knowledge of the diffuse chemosensory system in lamprey aids in understanding chemosensory stimuli detection and its influences on behaviours, which could be put to use in lamprey population management strategies. Future work could look at physiological and behavioural responses to stimulatory substances of spawner sea lamprey SCCs, specifically gill pore SCCs as chapter 2 data has shown that SCCs are the most abundant at that location compared to oral disc and tail locations. Additional

experiments on the biochemical characterization of SCCs could be performed to elucidate the neurotransmitter involved in fish SCCs synapses and to study the genome of the lamprey to probe for protein involved in the SCC transduction pathway.

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APPENDIX A: Protocols and solutions

Scanning electron tissue preparation

5% Gluteraldehyde fixative (also known as modified Karnofsky's fixative)

Stock solution of sodium cacodylate

((CH₃)₂AsO₂Na · 3H₂O; MW = 214.03)

0.2M Sodium cacodylate

Dissolve 4.28 g / 100 mL deionized water

0.1M Sodium cacodylate buffer

Mix 50 ml 0.2M sodium cacodylate with 50mL deionized water

Gluteraldehyde fixative (for 100mL's)

50mL 0.2M sodium cacodylate

20mL 25% Gluteraldehyde

30mL deionized water

- 1) Add 50mLs 0.2M sodium cacodylate to a flask with a stir bar
- 2) Add the gluteraldehyde
- 3) Pore some of the water into the empty gluteraldehyde tinctures. Use a glass transfer pipette to remove all water and gluteraldehyde residue and add to the flask. Pour remaining water into the flask.
- 4) Mix for several minutes

Osmium Tetroxide, 2% in 0.1M cacodylate buffer

Stock 4%, 250mg OsO₄

Safety protocols should be followed with notices put on doorways and fume hoods.

Double nitrile gloves should be worn with fume-resistant goggles.

1) Run ampule under warm water to melt crystals

Solution should be made in a glass container big enough for the ampule to fit in and a strong screw lid. This container should fit into a secondary container. (preferably a brown glass bottle)

2) Open ampule carefully and pore stock osmium and glass ampule into 6.25 mL ultrapure water

3) Cover tightly and put in a secondary container away from light. Leave overnight to dissolve.

4) Add 7 mL 0.2M sodium cacodylate, and mix well.

Tissue preparation for Scanning Electron Microscopy

- 1) Tissue should be fixed in gluteraldehyde fixative. Selected tissues should be cut and prepared to fix on a metal stub. Tissue should be transferred to individual vials that are tall with a small diameter and posted fixed for 1-2 days.
- 2) Gluteraldehyde is removed from vials and 0.1M sodium cacodylate buffer added to remove residual fixative
- 3) The Buffer is removed and 0.1M 2% Osmium is added to just cover the tissue sample. This is left on ice for an hour, checking every 20 minutes.
- 4) Samples are removed from ice and left for another hour, checking for forming precipitate, (indicating an air leak in the snap lid)
- 5) The osmium is removed and emptied into a container for proper disposal. 50% ethanol is added to the top of the vial, samples must be kept submerged in ethanol after this point
- 6) 50% ethanol is removed after 10 minutes and 70% ethanol is added for 20 minutes
- 7) 70% ethanol is removed after 20 minutes and 85% ethanol is added for 20 minutes
- 8) 85% ethanol is removed after 20 minutes and 95% ethanol is added for 20 minutes
- 9) 95% ethanol is removed after 20 minutes and 100% ethanol is added for 20 minutes
- 10) Four changes of 100% ethanol every 20 minutes

Samples were transferred to labeled microporous specimen capsules in 100% ethanol and taken to the University of Western Ontario, London, ON. Tissue was critical point dried and placed onto metal stubs to be gold sputter coated, this made the tissue conductive. The samples were then stored in air tight containers with dririte until imaged at GLIER at the University of Windsor, on an Environmental SEM.

Immunohistochemistry

Phosphate Buffer (PB) and Phosphate Buffered Saline (PBS) and Phosphate Buffered Saline with Triton X-100 (PBS-T)

Make stock solutions of sodium phosphate monobasic ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$; MW = 137.99) and sodium phosphate dibasic anhydrous (Na_2HPO_4 ; MW = 141.96)

0.2 M Sodium Phosphate Monobasic
Dissolve 27.6 g into 1 L of deionized water

0.2 M Sodium Phosphate Dibasic
Dissolve 28.4 g into 1 L of deionized water

0.2 M PB:
Mix 190 mL of sodium phosphate monobasic and 810 mL of sodium phosphate dibasic for pH 7.4

0.1 M PB:

Add 500 mL of deionized water to 500 mL of 0.2 M PB

0.1 M PBS:

Add 8.0 g NaCl and 0.2 g KCl to 1 L of 0.1 M PB

Add 1g NaN₃ per 1 L solution

0.1 M PBS-T:

Add 1 mL of Triton X-100 to 1 L of 0.1 M PBS

4% Paraformaldehyde (PFA) for 250 mL

55 mL deionized water

10 g paraformaldehyde

- 1) Add PFA to deionized water
- 2) Heat to approximately 55 °C and stir solution for 10 minutes, then clear by adding NaOH chips (approximately 1 chip) while stirring. Add 5 M NaOH dropwise until clear
- 3) Solution is clear when dissolved
- 4) Cool solution (on ice if desired) to 10°C
- 5) Bring volume up to 125 mL by adding 70 mL of distilled water
- 6) Bring volume up to 250 mL by adding 125 0.2 M phosphate buffer
- 7) Check pH using pH strips and adjust to 7.4

Acetylated Tubulin Immunocytochemistry Protocol as a general label of neural tissue

Adapted from: Frontini, 2002 (MSc Thesis)

- 1) Rehydrate slides in 0.1M PBS with 0.1% Triton X-100 for 30-40 min
- 2) Incubate in primary antibody: monoclonal mouse anti-acetylated tubulin (6-11B, Sigma) 1/1000 in 0.1 M PBS plus 0.1% sodium azide at 4 °C for two days
- 3) Wash 3x in 0.1 M PBS for 20 min each
- 4) Incubate in secondary antibody: Alexafluor 488 antimouse IgG (A11001, Life Technologies Inc) diluted 1/100 in 0.1 M PBS plus 0.1% sodium azide overnight at 4 °C
- 5) Wash 3x in 0.1M PBS for 20 min each
- 6) Coverslip with Vectashield Hardset Mounting Medium or Vectashield Mounting Medium with dapi to label nuclei.

Calretinin and Phospholipase C Protocol: Probes to label SCCs

Adapted from: Barreiro-Iglesias et al., 2008C; Hansen et al., 2014

- 1) Rehydrate sections in 0.1M PBS plus 0.1% Triton X-100 4 times for 30 minutes each
- 2) Block with 5% Goat Serum in 0.1M PBS-T for 2 hours

- 3) Incubate with Calretinin polyclonal antibody produced in goat and rabbit (7699/3, 7697, Swant) OR Phospholipase C antibody (Jane Mitchell, U of Toronto) at 1/1000 in 0.1 M PBS plus 0.1% sodium azide in keeper at 4°C on shaker for 3 days
- 4) Rinse five times (48 min each) in PBS at 4°C on shaker
- 5) Incubate with goat antirabbit Alexafluor 568 IgG (A11011, Life Technologies Inc) diluted 1/200 in 0.1 M PBS plus 0.1% sodium azide overnight-to-two days at 4°C in keeper on shaker.
- 6) Rinse five times (48 min each) in PBS at 4°C on shaker
- 7) Coverslip with Vectashield Hardset Mounting Medium or Vectashield Mounting Medium with dapi to label nuclei.

5-HT Protocol: Probe for serotonin-immunoreactive cells and fibers
Adapted from: Frontini et al., 2003

- 1) Rehydrate sections in 0.1M PBS 10 minutes
- 2) Wash in acetone for 10 minutes at -20°C
- 3) Wash in 0.1M PBS for 10 minutes
- 4) Block with 10% Goat Serum in 0.1M PBS plus 0.1% sodium azide for 30 minutes on a shaker
- 5) Incubate with 5-HT polyclonal antibody (20080, Immunostar) at 1/5 000 in 0.1 M PBS-T plus 0.1% sodium azide in keeper at 4°C on shaker for three days.
- 6) Rinse three times (20 min each), then two times (1 hour) in PBS at 4°C on shaker
- 7) Incubate with goat antirabbit Alexafluor 568 IgG (A11011, Life Technologies Inc) diluted 1/200 in 0.1 M PBS plus 0.1% sodium azide for overnight at 4°C in keeper on shaker
- 8) Rinse three times (20 min each), then two times (1 hour) in PBS at 4°C on shaker
- 9) Coverslip with Vectashield Hardset Mounting Medium or Vectashield Mounting Medium with dapi to label nuclei.

APPENDIX B: Tables and graphs

Table 1. Sample information from all specimens used in thesis.

(Abbreviations: SPMM spawner migrant male, SPMF spawner migrant female, SPIM spawner immature male, SPIF spawner immature female, MM migrant male, MF migrant female, SM spawner male, SF spawner female). Note: transformer is a metamorphic stage, parasite is a juvenile stage, and spawner 1 and 2 are both ovulatory/spermiating stage lamprey.

Age Class	Date fixed/ Dissected	Weight (g)	Length cm
Spawner 1	2013		
Parasite			
Transformer 1	22-May-14	3.9g	14.5cm
Transformer 2	23-May-14	4.30g	15.5cm
Spawner 2	18-Jun-14	426.6g	48cm
SPMM1	28-Jun-14	358.7	53.5
SPMM2	28-Jun-14	255.3	49.7
SPMM3	28-Jun-14	254.4	48.2
SPMM4	28-Jun-14	165.5	43.3
SPMM5	28-Jun-14	289.2	50.5
SPMM6	28-Jun-14	269.7	50
SPMF1	28-Jun-14	256.3	49.7
SPMF2	28-Jun-14	266.3	46.5
SPMF3	28-Jun-14	276.9	50
SPMF4	28-Jun-14	70.8	32
SPMF5	28-Jun-14	162.9	42.2
SPMF6	28-Jun-14	X	45.5
SPIM1	28-Jun-14	212	47.5

SPIM2	28-Jun-14	235.1	48.5
SPIM3	28-Jun-14	161.6	45
SPIM4	28-Jun-14	227	39.5
SPIM5	28-Jun-14	158.7	40.3
SPIM6	28-Jun-14	270.1	54
SPIF1	28-Jun-14	241.4	48
SPIF2	28-Jun-14	215.4	48
SPIF3	28-Jun-14	283.7	54
SPIF4	28-Jun-14	158.5	39.5
SPIF5	28-Jun-14	294.2	54
SPIF6	28-Jun-14	160	46.5
Larvae 1	28-Jun-14	X	X
Parasite 1 (Male)	23-Oct-14	187.5	48.5
Parasite 2 (Male)	27-Oct-14	85.3	39
MM1	May 13 2015	204.5	46.5
MM2	May 13 2015	181	42.5
MM3	May 13 2015	231.9	50
MM4	May 13 2015	253.8	54
MM5	May 14 2015	213.5	51.5
MF1	May 13 2015	192.4	43.4
MF2	May 13 2015	280.5	52
MF3	May 13 2015	248.3	50
MF4	May 13 2015	193.2	48.5
MF5	May 14 2015	120.5	42

SM1	June 8 2015	165	38.3
SM2	June 8 2015	171	39.5
SM3	June 8 2015	175.7	43
SM4	June 8 2015	288	47.5
SF1	June 8 2015	X	X
SF2	June 8 2015	165.1	44
SF3	June 8 2015	182.5	43
SF4	June 8 2015	156.6	43
Parasite 1 (male)	Dec 3 2015	72g	33
Parasite 2 (male)	Dec 3 2015	79g	38

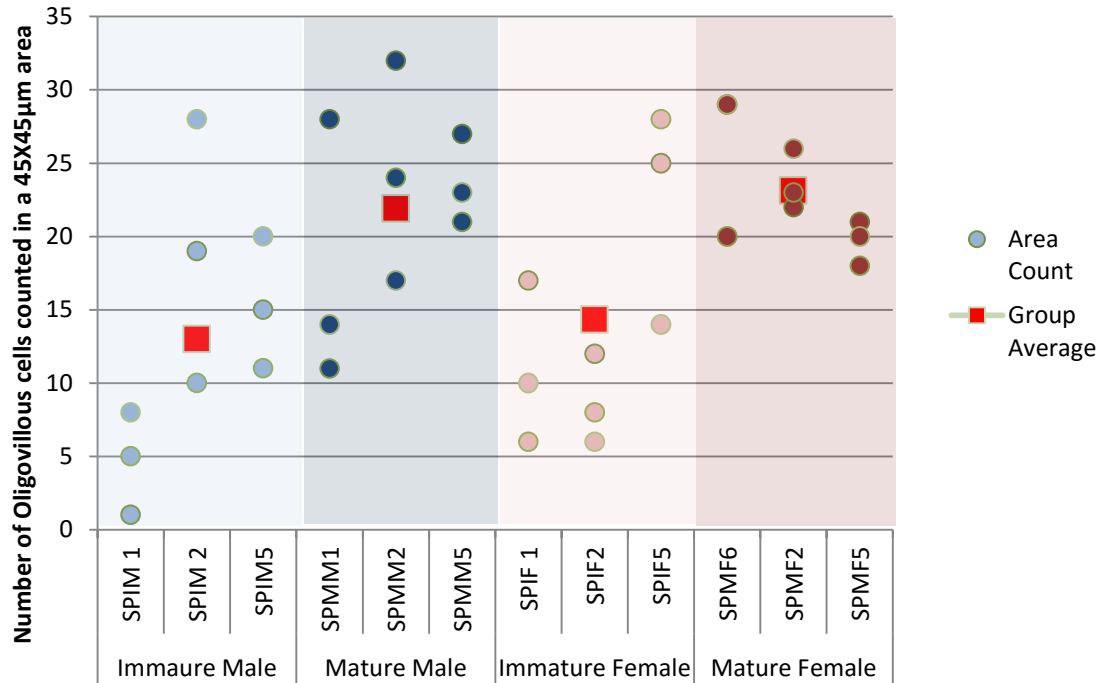
Table 2. Papillar SCC density in the gill pore of captured and matured spawning stage sea lamprey (2014).

First Field season 2014 - Sexually immature male and female sea lampreys were captured in traps from Lake Huron tributaries (United States Fish and Wildlife Service, Marquette, Michigan, USA) and held in 1000 L tanks supplied with Lake Huron water at ambient temperature at United States Geological Survey, Great Lakes Science Center, Hammond Bay Biological Station, Millersburg, Michigan, USA. Immature sea lampreys were placed in cages (1 m³) to mature in the Ocqueoc Rivers, Michigan, USA, and checked daily. Females were termed “ovulated” if eggs were released after manual pressure to the abdomen. Males were termed “spermiated” if they had a thick connective tissue dorsal rope and if milt was released after manual pressure. Ovulated females and spermiated males were removed from in-stream cages and returned to Hammond Bay Biological Station. Immature lamprey held in 1000L 10°C tanks and Ocqueoc river matured lamprey were then collected and brought to the University of Windsor for experimental use.

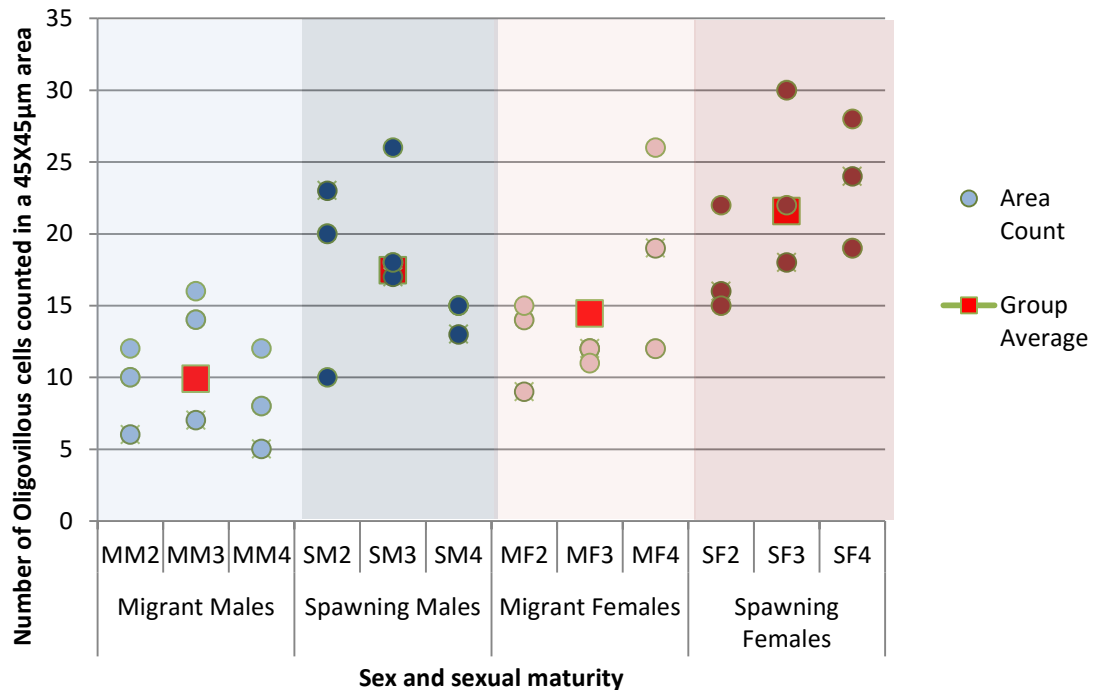
Immature males and females possessed more variation within their groups than mature males and females. Mature females possessed the most abundant amount of SCCs.
describe the experiment.

Group	Weight (g)	Length (cm)	Number of SCCs / 45 μ m ²	Group Average
Immature captured males	212	47.5	1, 5, 8	
	235.1	48.5	10, 19, 28	13
	158.7	40.3	11, 15, 20	
Matured spawning Males	358.7	53.5	11, 14, 28	
	255.3	49.7	17, 24, 32	21.89
	289.2	50.5	21, 23, 27	
Immature captured females	241.4	48	6, 10, 17	
	215.4	48	6, 8, 12	14.33
	294.2	54	14, 25, 28	
Matured spawning females	276.9	45.5	20, 29, 29	
	266.3	46.5	22, 23, 26	23.11
	162.9	42.5	18, 20, 21	

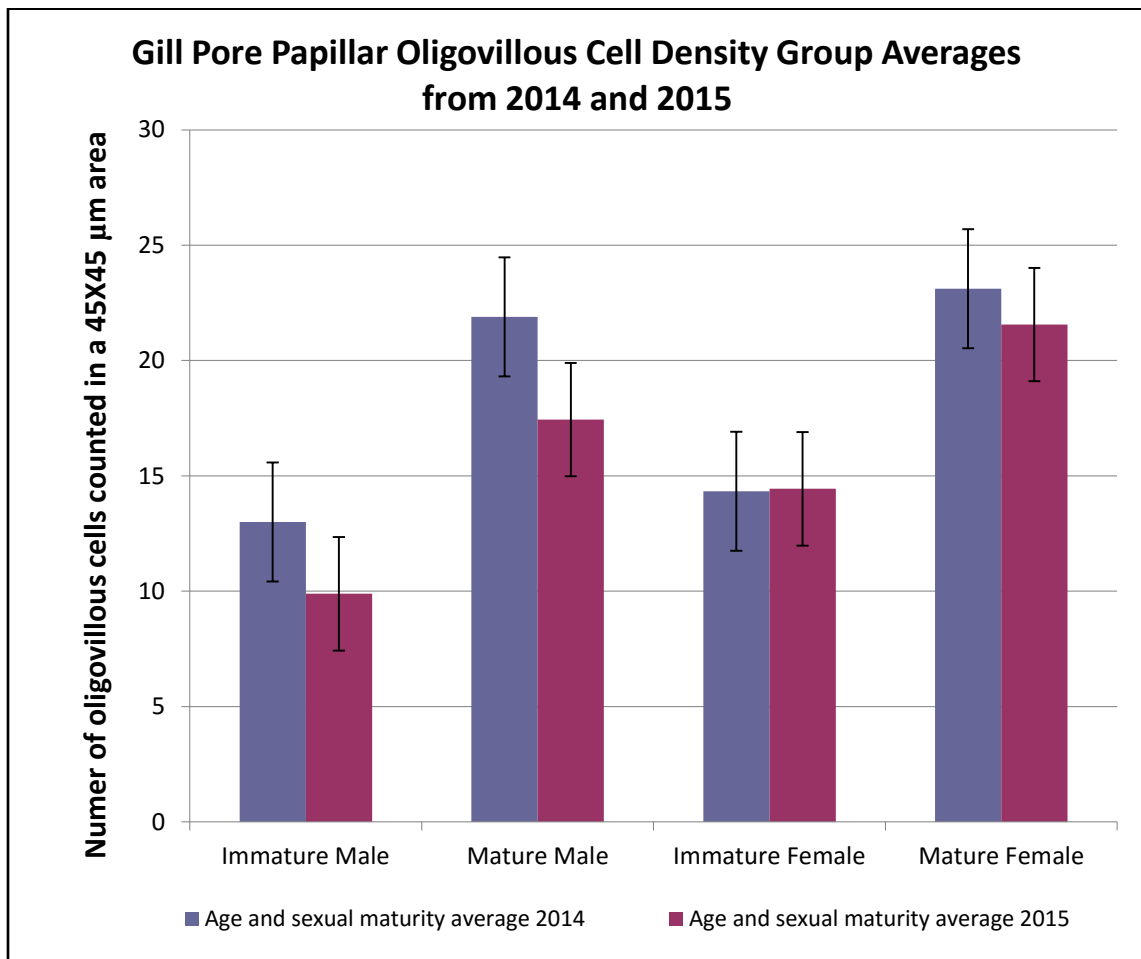
Papillar Oligovillous Cell Density (2014)



Papillar Oligovillous Cell Density (2015)



Graph 1 and 2. Papillar oligovillous cell density counts from 2014: artificially matured individuals and 2015: collected sexually immature migrants and sexually mature spawning lamprey. Three gill pore papillae per individual were counted for number of oligovillous cells/SCCs present in a $2025 \mu\text{m}^2$ area. This was repeated for three individuals in each group, being defined by sexually maturity status and sex. These were then averaged to calculate the “group average”. In both data sets, the mature females possessed the most abundant amount of cells counted; females also possessed more cells on average than males, regardless of sexual maturity status.



Graph 3. Gill pore papillae oligovillous cell density group averages from 2014: artificially matured individuals and 2015: collected sexually immature migrants and sexually mature spawning lamprey. Mature females possessed the most abundant amount of SCCs compared to males and immature females. This was found both in the artificially matured groups from 2014 and the actual migrants and spawning adults from 2015. In

2014 adult lamprey that had been previously captured were placed back into the Ocqueoc River to become sexually mature while the rest were held at a constant temperature which stops maturation. This is contrasted by 2015 samples where actual migrating lampreys from the deep lakes to the streams and rivers were collected and the following month sexually mature lamprey were collected directly from spawning nests.

APPENDIX C: Figures

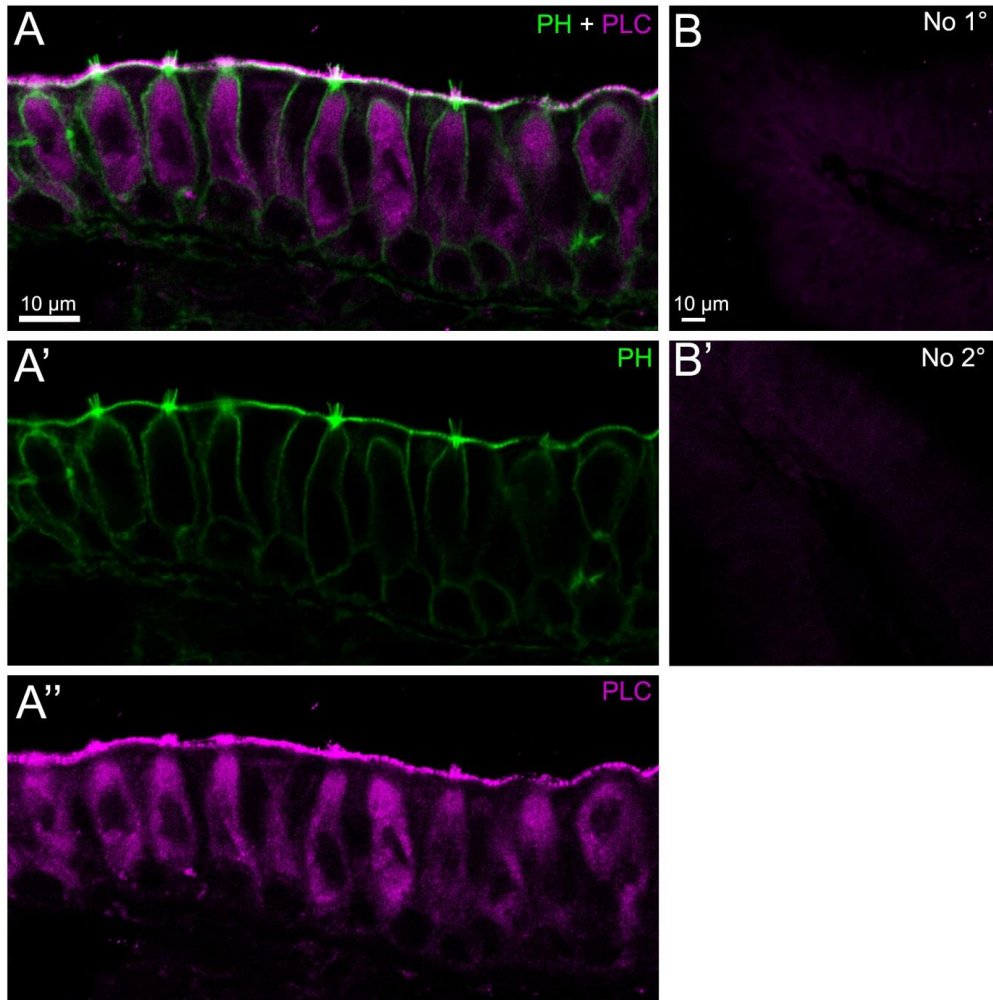


Figure 1. Phospholipase C labeling and negative controls of spawner gill pore papillae. A: Merge of phalloidin labeling in green (A') and phospholipase C labeling in magenta (A'').

B: Negative control. The phospholipase C antibody omitted to check for non-specific binding from the secondary antibody.

B': Negative control. The secondary antibody anti-rabbit Alexa Fluoro 568 was omitted to check for non-specific binding from the phospholipase C antibody.

Both show no labeling indicating that the labeling seen in A'' is specific.

APPENDIX D: 5-HT preadsorption experiment for antibody specificity in gill papillae

INTRODUCTION

Immunohistochemistry utilizes antibodies conjugated to antigens as a method of visualizing traits within sectioned or whole mount preparations. A preadsorption control is performed to verify that experimental labeling is specific to the antigen in question. The antibody being tested is fully saturated with its antigen before following immunolabeling protocols. A standard protocol is also performed as a control with the same dilution of antibody used in the preadsorbed solution.

MATERIALS AND METHODS

Antibody preadsorption and preparation

(This protocol is in part adapted from Paulk et al., 2009. Color processing in the medulla of the bumble bee (Apidae: *Bombus impatiens*). *J Comp Neuro.* 513(6):441-456.)

A 5-HT immunohistochemistry was prepared as a positive control for 5-HT labeling and a 5-HT preadsorbed with 5-HT solution was made to test for antibody specificity within tissue samples.

We thawed out a 5 μ l aliquot of 5-HT antibody (lyophilized whole serum, Immunostar, 20080). To prepare a 1/5000 anti-5-HT positive control solution, 1 μ l of 5-HT antibody was added to a plastic keeper with 5000 μ l PBS plus 0.1% sodium azide and labeled as '5-HT control'. The preadsorbing solution was prepared in a 1.5 ml autoclaved Eppendorf tube by adding 1 μ l 5-HT antibody to 5 mg 5-HT (Sigma, H-9523) and 500 μ l PBS plus 0.1% sodium azide and labeled as '5mg 5-HT preadsorbed'. This solution was

vortexed and allowed to incubate at room temperature on a shaker for 1 hour, then transferred to a shaker at 4°C overnight. The preadsorbed 5-HT solution was spun down at 14 500 RPM for 20 minutes at 4°C. The supernatant was collected and added to a plastic keeper with 4500 µl PBS plus 0.1% sodium azide.

Labeling protocol

Cryostat sections were collected from a spawner gill pore and prepared according to a 5-HT protocol adapted from Frontini et al. (2003). Briefly, slides were washed with PBS for 10 minutes, then 10 minutes in actone at -20°C followed by another PBS wash for 10 minutes at 4°C. Slides were blocked in a 10% goat serum in PBS for 30 minutes on a shaker at 4°C, then incubated on a shaker at 4°C in either the positive control 5-HT 1/5000 keeper or the 5mg 5-HT preadsorbed keeper overnight. Slides were washed in PBS for 2 hours then incubated in goat antimouse Alexafluor 568 IgG (Life Technologies Inc) diluted 1/200 in 0.1 M PBS plus 0.1% sodium azide overnight at 4°C on shaker. Slides were washed for a final time and coverslipped with Vectashield mounting medium.

RESULTS

Bright labeling was observed in both 5-HT immunoreactive fibers present in the connective tissue layers of the gill pore (Fig. 1A) and within SCCs on cross-sectioned papillae (Fig. 1B). In contrast to the control labeling, the 5mg preadsorbed slides showed no bright labeling in either the connective tissue layer (Fig. 1C) or in the cross-sectioned papillae (Fig. 1D).

DISCUSSION

The purpose of this control is to show that the labeling observed in a standard protocol is due to the antibody binding to its antigen. The 5-HT antibody preadsorbed with the 5-HT antigen showed no bright labeling indicating that most of the antibody was already bound to its antigen and did not bind to the antigen present within the tissue sample. This verifies that the labeling observed in Figure 1A and B is from the antibody binding to the proper antigen present within the tissue and not non-specific binding to another antigen.

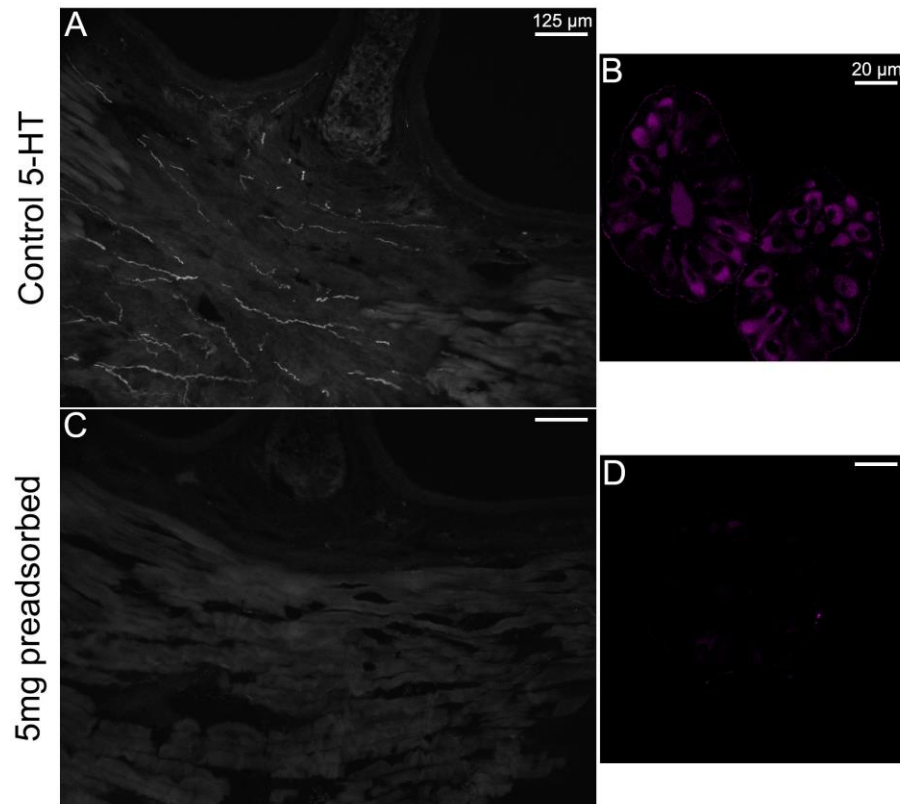


Figure 2. 5-HT pre-adsorption control of spawner gill pore and SCCs.

- A. Positive control of 1/5000 5-HT antibody in PBS immunolabeling protocol of the middle of a spawner gill pore. The structure in the center is the central process

and beneath that there are bright 5-HT immunoreactive fibers seen in white.

Epifluorescence microscopy

- B. Positive control 5-HT standard immunolabeling protocol of a cross-section of the tip of a papilla. Bright 5-HT immune-reactive cells can be seen along the periphery. Confocal microscopy
- C. 1/5000 5-HT antibody in PBS preadsorbed with 5mg 5-HT antigen tested on a spawner gill pore. The same view seen in A is shown in C, where the beneath the central process has no white brightly labeled fibers.
- D. 1/5000 5-HT antibody in PBS preadsorbed with 5mg 5-HT antigen tested on a spawner gill pore papilla cross-section. Almost no labeling is seen within the micrograph of a similar area to that shown in B.

Appendix F: Copyright permissions

Figures permissions

Figure 1.1

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To: "suntrest@uwindsor.ca" <suntrest@uwindsor.ca>

Dear Tina,

Thank you for contacting the Great Lakes Fishery Commission concerning the lifecycle image of the sea lamprey. Please let this email serve as permission for use of the lifecycle so long as you cite appropriately (as you did in the attached pdf).

Kind regards,

Ted Lawrence, PhD
Communications and Policy Associate
Great Lakes Fishery Commission
2100 Commonwealth Blvd. Suite 100
Ann Arbor, MI 48105
734.669.3008
www.glfc.org

Co- Authorship permissions

thesis copyright material

2 messages

Tina Suntres <suntrest@uwindsor.ca>

Wed, Apr 20, 2016 at 3:22 PM

To: Gheylen Daghfous < >

Hello Gheylen,

I am writing to ask permission, for copyright purposes, to include material collected with you in my final thesis. Your response will be included in the appendix.

Thank you

- Tina Suntres

Gheylen Daghfous < >

Wed, Apr 27, 2016 at 3:20 PM

To: Tina Suntres <suntrest@uwindsor.ca>

Hi Tina,

You have my permission to use material collected together in you thesis.

Gheylen

thesis copyright permissions

2 messages

Tina Suntres <suntrest@uwindsor.ca>

Wed, Apr 20, 2016 at 3:23 PM

To: Rejean Dubuc <>

Hello Dr. Dubuc,

I am writing to ask permission, for copyright purposes, to include material collected with you in my final thesis. Your response will be included in the appendix.

Thank you

- Tina Suntres

Réjean Dubuc <>

Thu, Apr 21, 2016 at 9:47 AM

To: Tina Suntres <suntrest@uwindsor.ca>

Dear Tina,

There is no problem for you to include material collected with us in your thesis.

Best wishes,

Réjean

Réjean Dubuc Ph.D.

Directeur

[GRAPA](#) | Groupe de Recherche en Activité Physique Adaptée

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copyright permissions

2 messages

Tina Suntres <suntrest@uwindsor.ca>

Wed, Apr 27, 2016 at 10:28 AM

To: Barbara Zielinski <>

Hi Dr. Zielinski,

I am writing to ask permission, for copyright purposes, to include material collected with you in my final thesis. Your response will be included in the appendix.

Thank you

- Tina Suntres

Barbara Zielinski <zielin1@uwindsor.ca>

Wed, Apr 27, 2016 at 10:31 AM

To: Tina Suntres <suntrest@uwindsor.ca>, Barbara Zielinski <>

I grant my permission for the M.Sc. thesis of Tina Suntres to include material collected with me.

VITA AUCTORIS

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2010-2014

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