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Studies Of Cells Within Three Chemosensory Structures In The Sea Lamprey (Petromyzon marinus)

Zeenat Aurangzeb
University of Windsor

STUDIES OF CELLS WITHIN THREE CHEMOSENSORY STRUCTURES

IN THE SEA LAMPREY (*Petromyzon marinus*)

By

Zeenat Aurangzeb

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by

Zeenat Aurangzeb

APPROVED BY:

_____

A. Hubberstey

Department of Biomedical Science

_____

O. Love

Department of Integrative Biology

_____

B. Zielinski, Advisor

Department of Integrative Biology

December 17, 2019

Declaration of Originality

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Abstract

Chemosensory systems play an important role in any organism collecting and processing information to regulate behaviours such as feeding, homing, avoidance and reproduction. In this thesis, I investigated the cells within the olfactory system and the diffuse chemosensory system of the sea lamprey. Calcium imaging of olfactory epithelial cellular responses to the polyamine odorants spermine and spermidine showed that separate cells responded to these odorants. The secondary goal of this thesis was focused on the loading of cells in the accessory olfactory organ (AOO) with neural activity indicators to examine the odorant response profiles of these cells. Multiple *in vivo* and electroporation dye loading strategies were attempted, with no successful loading of these cells. Investigating the AOO is still critical as it may provide insight into the importance of having more than one olfactory pathway. The third goal of this study was to investigate the location of nerve fibers on dermal papillae that contain solitary chemosensory cells (SCCs) of the diffuse chemosensory system. The findings show that nerve fibres entered the dermal papillae and, in many cases, were seen adjacent to SCCs in oral larval papillae and in nasal, gill and fin papillae following metamorphosis and in adults. This finding indicates that SCCs communicate with nerve fibers located in the cutaneous dermal papillae and that these fibers may be involved in processing sensory information that stimulates specific behavioural responses. Overall, this thesis examines the function of specific cells associated with lamprey chemosensory systems which may provide insight to appropriate behavioural responses necessary for survival and reproduction.

This work is dedicated to my mother, Rubina and my father, Aurangzeb, to my daughter, Parishay, to my sisters and my brother.

Thank you for their help, patience, love and inspiration

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List of abbreviations

3kPZS	3-keto petromyzonol sulphate
ACA	Allocholic acid
Arg	Arginine
AOO	Accessory olfactory organ
Arg	Arginine
cAMP	Cyclic adenosine monophosphate
CR	Calretinin
DKPES	3,12-diketo-4,6-petromyzonene-24-sulfate
EOG	Electro-olfactogram
GPCR	G protein coupled receptor
His	Histidine
MLR	Mesencephalic locomotor region
MOE	Main olfactory epithelium
OB	Olfactory bulb
OE	Olfactory epithelium
OR	Odour receptor
OSN	Olfactory sensory neuron
PFA	Paraformaldehyde
PH	Phalloidin
PZS	Petromyzonol sulphate
SCC	Solitary chemosensory cell
Spd	Spermidine
Spm	Spermine
TAAR	Trace amino acid receptor
TCA	Taurocholic acid
VNO	Vomeronasal organ

Chapter 1: General introduction and overview

1.1 General overview

Organisms are surrounded by numerous stimuli in a continuously changing environment. These stimuli help organisms to find food, hosts, mates and ovipositional sites and avoid predators and pathogens. Rats sense danger after smelling the fox urine component 2-phenylethylamine and will actively run away from the odorant (Ferrero et al., 2011; Brechbühl et al., 2013). Feeding behaviour is usually stimulated by amino acid odorants in many fish species (Carr et al., 1977; Sorensen et al., 1998; Rolen et al., 2003) while peptides associated with the multiple histocompatibility complex signal kin recognition in fish and mice (Hinz et al., 2013). These stimuli are perceived and processed by different sensory systems including the visual, auditory, olfactory, taste and diffuse chemosensory systems. These systems consist of many different sensory neurons that detect the external stimuli and subsequently transmit signals to produce a behavioural response. Understanding how different chemosensory systems work in an organism is critical as it allows us to comprehend how the organisms thrive in their niches.

The sea lamprey (*Petromyzon marinus*) is a fascinating species to study the chemosensory systems and is an experimental model for this study. As a living descendant of the earliest craniate, the study of the lamprey provides an opportunity to study the major evolutionary transitions between craniate and vertebrate (Stensio, 1968; Forey & Janvier, 1993, 1994). In other words, we can explore their evolutionary history, including their shared common ancestry and their phylogenetic lineage.

The study of sea lamprey's chemosensory system is essential because of its parasitic nature affecting native fish populations, including salmon and trout, in turn impacting the billion-dollar fishing industry in the Great Lakes (Fetterolf, 1980) resulting in a need for population control strategies (Applegate, 1950). Studying the chemosensory systems may provide an understanding

of how the sea lamprey flourish in the Great Lakes and further using that information to develop an applicable method for controlling the sea lamprey using either pheromones, amino acids or polyamines (Li et al., 2013; Scott et al., 2019; Johnson et al., 2015). Each life cycle phase of the sea lamprey (larva, metamorphosis [transformer], juvenile adult parasite and adult spawner) serves a specific yet crucial role for their survival and reproduction (Figure 1.1). During the larval phases, the sea lamprey filter feed the food such as algae and other microscopic organisms (Smith & Tibbles, 1980; Manzon et al., 2015); they also release migratory pheromones to attract spermiating males (Li and Sorensen, 1997; Siefkes & Li, 2004; Johnson et al., 2009). During the metamorphosis and juvenile stage, they move around the upstream river, navigate the surrounding environment and use food cues to find food and other chemicals to avoid predator (Purvis, 1980; Potter et al., 1982; Youson, 2003). During the adult spawner stage, they cease eating and migrate upstream toward the breeding area (Walaszczyk et al., 2013; Moore & Schleen, 1980). Spawner sea lamprey migrate up stream by detecting the migratory pheromones released by the larva (Li and Sorensen, 1997; Siefkes & Li, 2004; Johnson et al., 2009). Adult spawner males build their nest (Li et at., 2005, 2003; Siefkes et al., 2005) and release pheromones to attract ovulating females to their nest to lay the next generation of eggs and then the spawners themselves die. Thus, all phases of sea lamprey are dependent on sensing odorants in their environment to survive and reproduce, making it critical to develop an understanding of how the chemosensory system is attributed to their behaviours.

The objective of this thesis is to gain an understanding of the function of specific cells within three chemosensory structures: the main olfactory epithelium, the AOO, and the dermal papillae that contain solitary chemosensory cells (SCCs). The general focus of the first study is to test if individual olfactory sensory neurons in the main olfactory epithelium detect specific

odorants or multiple odorants. It has been previously investigated that amino and bile acids stimulate specific behaviours in sea lamprey (Li et al., 1995, 2002; Li & Sorensen, 1997; Siefkes & Li, 2004; Sorensen et al., 2005; Johnson et al., 2009, 2005; Siefkes et al., 2003) and that individual olfactory epithelial cells respond to specific odorants (Grande, 2017). It has yet to be investigated if two very similar molecules, such as spermine and spermidine, stimulate individual cells or multiple cells. These odorants may be involved in eliciting behavioural responses related to reproduction in the adult sea lamprey or other response such as stress response (Scott et al., 2019; Tabor & Tabor, 1985; Takahashi et al., 2010). Thus, it is important to investigate the stimulatory properties of both spermine and spermidine on olfactory sensory neurons of the sea lamprey. In this study, the spermine and spermidine responses were tested on main olfactory epithelium at larval stage of sea lamprey. Larval stage of sea lamprey was used to test odorant responses because it is mentioned in many studies that fishes start learning to recognize natal stream odours before the emergence of feeding period and the earlier epithelia within the olfactory pit mimics of those of adult olfactory epithelia (Dittman et al., 2015; Zielinski & Hara, 1988; Lorenz, 1935;Yanagi et al., 2004). The study done by Zielinski et al., (2005) showed that the main olfactory epithelium of pro-larval phase sea lamprey responded to L-arginine, taurocholic acid, petromyzonol sulfate (a lamprey migratory pheromone), and water conditioned by conspecifics. Thus, the main olfactory epithelium of larval phase sea lamprey may reflect the odorant response pattern like the adult olfactory epithelia. The secondary goal of the study is to load the olfactory sensory neurons in AOO with a dye indicator for optical calcium imaging. While calcium imaging in the main olfactory epithelium are currently under investigation, the odorant response characteristics of sensory neurons in the AOO are not known. The cells of the AOO are connected to the external environment through the main olfactory epithelium via a

duct; these cells are involved in eliciting motor behaviours in the sea lamprey (Derjean et al., 2010). Examining the characteristic responses of cells in the AOO may explain the importance of having two distinct olfactory subsystems, even with both olfactory subsystems detecting overlapping odorants (Green et al., 2017; Derjean et al., 2010). To study the characteristic of OSNs within AOO, it is essential to strategize the method to load the cells with the dye. In the study, the loading of dye in cell was done in all phases as it is unknown which stage provide precise loading. The sea lamprey has another chemosensory system, the diffuse chemosensory system, (Whitear, 1992) which has been reported having specialized cells, referred as solitary chemosensory cell (SCCs). These SCCs are only located on the oral dermal papillae in the larvae sea lamprey, whereas, they are located on the oral disc, nostril, gills and dorsal fin papillae after the metamorphic transformation phase (Suntres et al., 2019; Daghfous et al., 2019). The third goal of this study was to investigate the innervation of epithelium on papillae during specific stages of the sea lamprey life cycle. The presence of nerve fibre near SCCs may indicate that the SCCs are involved in sending sensory information to the central nervous system (Saunders et al., 2014; Barreiro-Iglesias et al., 2008) where the information is processed in order to produce appropriate response.

1.2 Olfaction and the olfactory subsystems

The olfactory system is a well-studied sensory system in different fish. it is responsible for discriminating and detecting odorants such as amino acids (Hara, 1975,1982), sex steroids (Resink et al., 1989; Cardwell et al., 1992; Sorensen et al., 1992; Nilsson et al., 2015), bile acids (Cardwell et al., 1992; Sorensen et al., 1987; Studer et al., 2012) and other compounds (Sutherlin & Sutherlin, 1971; Hara, 1975). These different odorants provide information to organisms to produce specific behaviour related to reproduction, food and survival (Hildebrand & Shepherd,

1997). Thus, the question is, how is an organism able to detect an odorant stimulus and respond?

In amphibians and rodents, the olfactory system consists of different olfactory subsystems responding to odorants (Barrios et al., 2014; Liberles et al., 2014; Taniguchi et al., 2011). Rodents have multiple spatially distinct olfactory subsystems: the main olfactory epithelium (located on nasal turbinates), the vomeronasal organ (situated on the rostral nasal base of nasal septum), the septal organ of Masera (located near the base of nasal septum rostral to nasopharyngeal canal), and the Grueneberg ganglion (located in submucosa of the main olfactory epithelium lining the rostral tip of nasal septum) (Munger et al., 2009; Levai & Strotmann, 2003; Storan & Key, 2006). These subsystems detect both different and overlapping odorants, relaying these signals to different regions of the olfactory bulb to stimulate specific behaviours (Liberles et al., 2014). In mammals, the main olfactory epithelium detects volatile odorants, and these sensory neurons send the information to the main olfactory bulb (Liberles et al., 2014), whereas, the vomeronasal organ detects non-volatile odorants, including pheromones and this sensory information, is processed in the accessory olfactory bulb (Restrepo et al., 2004; Spehr et al., 2006; Slotnick et al., 2010). The neural responses of different olfactory subsystems project from the olfactory bulb to specific higher brain regions, which are associated with behaviours (Restrepo et al., 2004; Spehr et al., 2006; Slotnick et al., 2010; Munger et al., 2009).

The sensory cells found within these olfactory subsystems are called olfactory sensory neurons (OSNs), these OSNs are bipolar and are the only neurons in the body that are directly exposed to both the external environment and the internal central nervous system (the axons terminate in the olfactory bulb). From various studies it is known that the numerous OSNs within these olfactory subsystems are different with respect to morphological characteristics, odour receptor proteins, signal transduction cascades and ion conductance channels (Hildebrand &

Shepherd, 1997; Kay & Stopfer, 2006). In rodents, these cells are ciliated in the main olfactory epithelium and microvillous in the vomeronasal organ (Liberles et al., 2014; Munger et al., 2009). In teleost fish, the OSNs are either ciliated or microvillous in the single olfactory epithelium with no other olfactory subsystems (Zielinski & Hara, 2006; Hansen & Zielinski, 2005; Sato & Suzuki, 2001) while in the sea lamprey, all OSNs are ciliated in both the main olfactory epithelium and AOO (Ren et al., 2009).

In mammalian, teleost, and sea lamprey, the OSNs express different types of G-proteins coupled with seven transmembrane receptors (GPCRs). These include odour receptor (OR)-type receptors, vomeronasal type 1 (VR1) and vomeronasal type 2 receptors (VR2) (Buck & Axel, 1991; Firestein, 2001; Libants et al., 2009; Munger et al., 2009). In rodents, some express the non-GPCR receptors, too, named MS4As (Greer et al., 2016). In rodents, OR-types receptors are found in the main olfactory epithelium, and VR1 and VR2 receptors are expressed in the vomeronasal organ (Munger et al., 2009). The MS4A is found within the recesses region of the main olfactory epithelium (Greer et al., 2016). More than 1000 GPCR olfactory receptor genes have been identified in mice, but each OSNs expresses only a single olfactory receptor (Mombaerts et al., 2004; Glusman et al., 2001; Young et al., 2002; Zhang et al., 2002). However, most of the studies indicated that the receptors used a combinatorial code system to recognize a vast range of odorants (Malnic et al., 1999; Nara et al., 2011), explaining the ability of the olfactory system to discriminate between many different odorants. For example, one odorant may be recognized by four receptor proteins, and three receptors may recognize another odorant with slightly different structures at a specific concentration. Thus, two odorants may activate the same receptors and, activate slightly different receptors due to their structural differences and similarity. Therefore, the individual receptors are broadly tuned in the olfactory

system of studied species and the receptors can recognize odorants structural similarity and differences at given concentrations. Malnic et al., (1999) also found that a change in the concentration of odorants resulted in a shift in the "code" assigning the quality of odour sensing. For example, if the concentration of the odorant changed, the odorant may start binding to different receptors in addition to its original receptor; the specificity of receptors to odorants changes at different concentrations.

The odorant receptors undergo a conformational change upon binding to the odorant, the receptors interact with G-coupled proteins activating the secondary messengers, such as cyclic adenosine monophosphate, inositol-(1,4,5)-triphosphate or cyclic guanosine monophosphate. These secondary messengers (such as cAMP) activate a cyclic nucleotide-gated channel causing an influx of Ca^{+2} ions into the cell from the extracellular fluid into nasal cavity within the OSNs. This increased Ca^{+2} ion concentration inside the OSNs changes the resting membrane potential, resulting in depolarization in the cell (Munger et al., 2009; Figure 1.2).

To understand if the receptors are using the combinatorial mechanism in any given species, various experiments can be conducted such as the cross-adaption experiment and calcium imaging. Calcium imaging takes advantage of the Ca^{+2} ion influx happening in OSNs to determine the receptors ability to bind to different odorants and therefore providing the location of OSNs undergoing a calcium concentration increase during odorant responses can be examined Leinders-Zufall et al., 2000; Schild et al., 1995; Sansone et al., 2014). For example, Sanz et al., (2005) used calcium imaging to test 100 odorants against the human odorant receptor, named class 11 OR1G1, which was expressed in transfected cells and it was found that this receptor was broadly tuned toward 9-10 carbon chain length compounds with diverse functional groups in humans. Nara et al., (2011) found that half of the mouse OSNs were narrowly tuned to one

odorant (43 out of 76 OSNs) or two to three odorants (26 out of 73 OSNs). These receptors that were narrowly tuned to two to three odorants had similar structures such as, the α-farnesene compound that was a male mouse pheromone, or individual musk odorants or the fecal odorants indole and skatole. Using calcium imaging, Leinders-Zufall et al., (2000) discovered that six putative pheromones activate unique non-overlapping subsets of OSNs in an intact vomeronasal organ and that these receptors have highly selective tuning properties using calcium imaging. It was also found that if the pheromone concentration was increased, the tuning curve did not broaden, unlike olfactory sensory neurons in the main olfactory epithelium. These narrowly tuned properties in receptors may indicate a tight connection with the olfactory cue and the regulation of specific innate behavioural response in vomeronasal.

The sea lamprey has two olfactory subsystems, main and accessory subsystems (Ren et al., 2009; Derjean et al., 2010), the axons of OSNs from the main olfactory epithelium project into the olfactory nerve and extend into the non-medial portions of the olfactory bulb. The axons from the AOO enter only the medial region of the olfactory bulb and converge into one spherical medial glomerulus (Green et al., 2017; Figure 1.3). Derjean et al., (2010) showed that projections from the medial region of the olfactory bulb stimulate the hindbrain mesencephalic locomotor region, which is a motor control center; thus, the medial region may be involved in stimulating general locomotion behaviour in sea lamprey. Axons from the non-medial area are projected to the lateral pallium and the extra pallial forebrain (Derjean et al., 2010; Green, 2012) (Figure 1.3), the non-medial region is also gated by GABAergic neurons, which inhibit locomotion in the sea lamprey (Daghfous et al., 2018); thus, the non-medial regions maybe involved in discriminating between different odorants and sending the signal to different parts of a higher brain structures to elicit specific behaviour. The projections are being send to olfactory bulb and higher brain

structure at specific regions, but it is also necessary to know what is happening in main olfactory epithelium and AOO. The individual OSNs within main and accessory subsystems may be binding to specific odorant or more than one odorant in sea lamprey, observed in higher organisms.

Electro-olfactogram (EOG) recording have demonstrated that the adult sea lamprey is capable of distinguishing larval bile acids from other bile acids (Li et al., 1995). In EOG method, the electrode was placed at a specific location and measured response magnitude from many OSNs in the main olfactory epithelium using a range of concentrations to build the concentration-response curve; from this experiment. By studying the concentration-response curve of three different bile acids (allocholic acid, petromyzonal sulphate and taurolithocholic acid 3-sulphate). It could be determining that each bile acid had different concentration-response curve. The cross-adapting experiment on the main olfactory epithelium also showed that there was no change in EOG responsiveness of the epithelium when either of bile acid was applied (Li et al., 1995). The EOG method could not show which OSNs were activated in real-time. On the other hand, calcium imaging experiments on OSNs in the main olfactory epithelium could indicate which OSNs bind to which odorant in real-time. A study done by Grande, (2017) suggested that the OSNs within the main olfactory epithelium are very sensitive and specific to different putative pheromones and that separate cells respond to a particular odorant. It has yet to discover if the OSNs of sea lamprey also show specificity to another class of odorants, the polyamines. It has been hypothesized that the individual OSNs of the main olfactory epithelium may respond to specific odorants in sea lamprey.

Very limited information is known about the cell's characteristics within the AOO; however, it is known that the axons of the OSNs from the AOO converge into a single

glomerulus in the olfactory bulb. The blub recording indicated that both the main and accessory olfactory subsystems may detect overlapping odorants (Derjean et al., 2010; Green et al., 2017). It is vital to investigate whether the individual cells in the AOO show specificity to any odorants or not. The difficult location and small size of the AOO make EOG recording futile, in lieu calcium imaging experiments may help to measure the cell responses to odorants within the AOO. To characterize the cells in the AOO and understand how the AOO works at the cellular level, loading of the calcium imagining indicator dye needs to be very methodical and precise.

1.3 The diffuse chemosensory system

The diffuse chemosensory system has the capabilities of detecting chemicals within the lamprey's environment and producing a specific behaviour. This system includes specialized cells known as the solitary chemosensory cells (SCCs) (Kortschal, 1996) or have also been referred as oligovillous cells by Whitear & Lane, (1982). The SCCs are characterized by their spindle-shape with prominent microvilli at apex. Microvillous are supported by actin filaments and a single nerve fibre approaches to the base of SCCs (Whitear, 1965, 1992; Finger et al., 2003). The SCCs have been found in the epidermis of some aquatic vertebrates, including the sea lamprey (Kortschal, 1996; Whitear, 1992; Daghfous et al., 2019) as well as many ammonitic marine animals such as shark skin (Peach, 2005), and in the skin and gills of teleosts (Finger, 1997; Lane & Whitear, 1982). In mammals, SCCs are found in the nasal respiratory epithelium in mice (Finger et al., 2003) and within the respiratory tract, larynges, and trachea of cows (Tizzano et al., 2006). In the brook lamprey (*Lampetra planeri*) and the European river lamprey (*Lampetra fluviatilis*), SCCs have been found on protruding papillae, the oral disc, nasal pores, gill pores and dorsal tail fins (Whitear & Lane, 1983).

SCCs are secondary receptor cells that differ with respects to the locations, innervation patterns, and functions in the few species that have been studied according to Finger, (1997). In mammals, SCCs are found to be innervated by the trigeminal nerve (Finger et al., 2003), whereas in sea robins the SCCs are innervated by spinal nerves located on the rays of the pectoral fin (Morril, 1895). In rocklings, the SCCs are innervated by the recurrent branch of the facial nerve on the dorsal anterior fins (Kotrschal et al., 1984, 1988; Whitear & Finger, 1993b). However, the sea lamprey does not follow any of the above innervations with the SCCs on the gill papillae being innervated by the glossopharyngeal or vagus nerves (Daghfous et al., 2015).

The role of SCCs in many species is not clear; it has been suggested by Finger et al., (1997) that these cells may aid in detecting feeding cues and promoting foraging behaviour in species. A potential role of SCCs may be the ability to discriminate the presence of conspecifics and non-conspecifics, utilizing alarm substances found in fish body mucus (Pfeiffer, 1963; Kortschal, 1996; Chia et al., 2019). According to Chia et al., (2019), the skin of aquatic species produces alarms substances that may induce an aversive behaviour in species (Peters et al., 1991; Finger, 1997). SCCs have shown discrimination of odorants by sampling the water samples. The sea robin and rockling detect fish body mucus and bile acids for their surrounding water (Whitear, 1992; Kotrschal, 1995, 1996). The amphibious desert toad (*Bufo alvarius*) uses these cells to discriminate salt tastes (Nagai et al., 1999), while in mammals, the SCCs respond to bitter irritants to induce an inflammatory response (Sundres et al., 2014). It has been found that the bitter irritants bind to the G-coupled receptors and activate inositol trisphosphate (IP_3) through phospholipase C mediated cascade pathway, this in turn causes a calcium influx in the cells. This calcium influx signals the TRPM5 channels to open, depolarizing the cells to release acetylcholine onto nociceptive fibres, causing an inflammatory response.

Little is known about the function of the diffuse chemoreception system in comparison to the olfactory system and its roles in initiating motor responses in the sea lamprey (Daghfous et al., 2012). The only physiological study done on lamprey is the electrophysiological recordings of the nerve fibres below the oral disc papillae of *Lampetra planeri* (Baatrup & Doving, 1985) that the SCCS responded to acetic acid, sialic acid, fish body mucus, and dead trout water washings, water that contain dead trout (Baatrup & Doving, 1985) suggesting a possible role in feeding behaviour.

In sea lamprey, the SCCs are found on the dermal papillae of the oral disc, nasal pore, gill pores, anterior and posterior dorsal fins (Figure 1.4, Daghfous et al., 2019). The abundance of SCCs seems to increase with papillae numbers, which in turn increases as the lampreys develop into the later stages (Suntres, 2019; Daghfous et al., 2019). The labelling of cells with a calretinin antibody in the gill's papillae showed the presence of intracellular calcium, suggesting that the cells may play an essential role in signalling (Villar-Cheda, 2006). It has been discovered that the SCCs, found in gill pores of sea lamprey share biochemical properties with gustatory taste cells, and the solitary chemosensory cells of teleost, reptile and mammalian (Barreiro-Iglesias et al., 2008; Hansen et al., 2014; Finger et al., 2003; Saunders et al., 2014; Tizzano et al., 2006; Hansen et al., 2007; Suntres, 2016). Suntres, (2016) confirmed that the nerves found within the gill's papillae (Daghfous et al., 2015) are innervating the SCCs; she labelled the papillae with the antibody acetylated tubulin which is a marker for detecting neuronal tissue (Frontini et al., 2003; Barreiro-Iglesias et al., 2008). It has also been reported that the trigeminal nerve innervated the nose and oral papillae, while the dorsal fin papillae were innervated by dorsal spinal roots (Daghfous et al., 2019). It is yet to confirmed if the above-mentioned nerve fibres that were found within the nostril, oral disc and fins papillae are associated with SCCs as these nerve fibres

may be related to Merkle cells or other types of receptors cells, according to Daghfous et al., (2019). To find whether the nerve fibres found within the papillae are innervated with SCCs, immunohistochemical techniques can be implemented to label the nerve fibres with the antibody acetylated tubulin, previously used by Suntres, (2016). Each stage of the sea lamprey life has a very different behavioural role, ranging from filter-feeding, to active parasitic feeding, to migrating and spawning therefore, it crucial to find the presence of SCCs and their innervation in each regions, oral disc, fins, nostril and gills at specific phases of sea lamprey. Understanding of diffuse chemosensory system may provide another platform to prevent expansion of sea lamprey in the Great Lakes.

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Figures

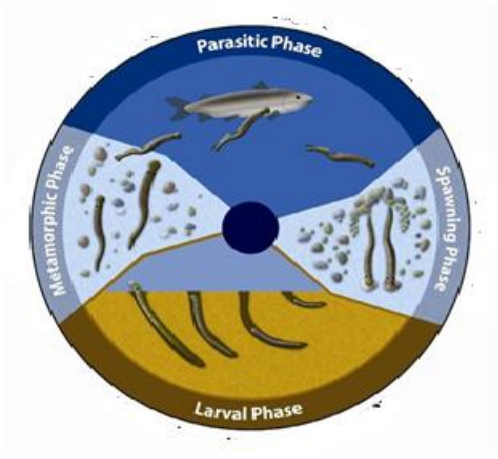

Figure 1.1: The Sea lamprey life cycle

The sea lamprey begins its life as a larval filter feeder living primarily in sand, it then transforms marking the metamorphic phase and migrates downstream where it remains during the summer. Following this period, they enter the parasitic phase where the lamprey feed on different fish species increasing their body size as they transform into the adult stage. When they become sexually mature, they move upstream to find a suitable spawning area where eggs are laid, and the lamprey die. (Image adapted from the Great Lakes Fishery Commission website, http://www.glfc.org/sealamp/images/sea_lamprey_life_cycle.jpg)

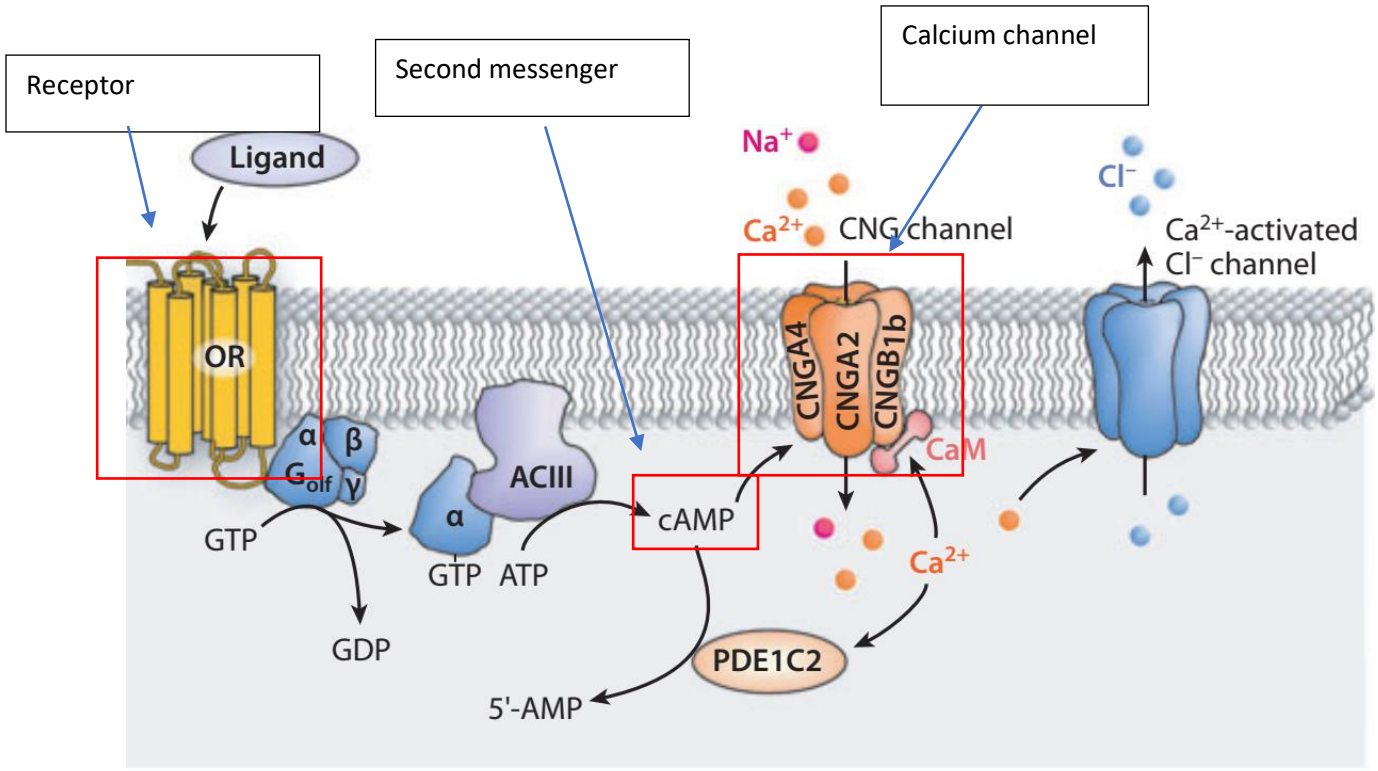

Figure 1.2: Activation of second messenger, cyclic (AMP) and the opening of calcium channels upon ligand binding to the receptor

When the ligand binds onto the olfactory receptor (OR) it activates the G-protein coupled receptor (GTP) resulting in the release of an α subunit. The α subunit activates adenylyl cyclase (AC111) converting adenosine triphosphate into cyclic adenosine monophosphate (cAMP) which is responsible for opening the calcium ion channels (CNG channel) allowing Ca^{+2} or Na^{+} to enter the cell resulting in the depolarization of the sensory neurons. (adapted from Munger et al. 2009)

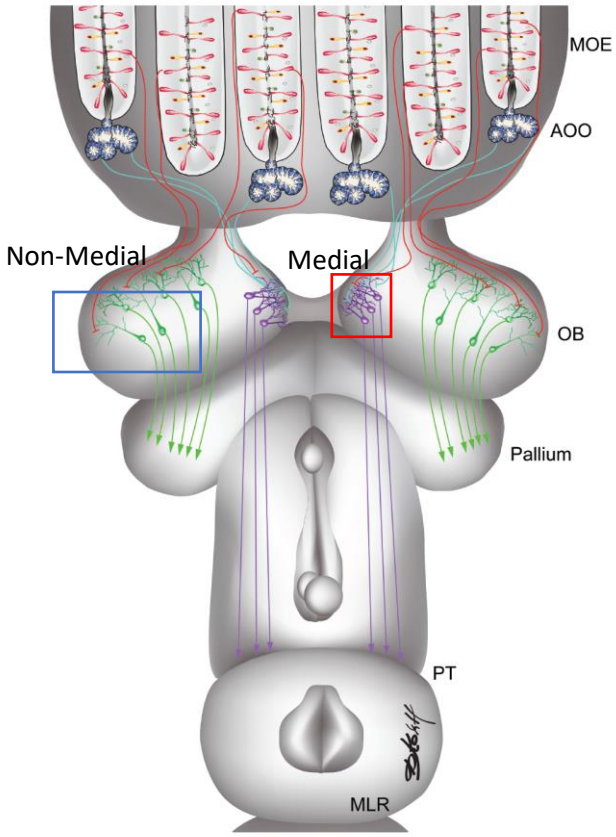

Figure 1.3: The Olfactory sensory neurons input pathway in the sea lamprey

The medial region (red box) of the olfactory bulb receives sensory inputs from the AOO (light blue) which then sends information to posterior tuberculum (PT) (purple) and further onto the mesencephalic locomotor region (MLR) connecting the AOO to the motor control centre. The information coming from the main olfactory epithelium (red axons) that enters the non-medial (blue box) regions of the olfactory bulb, projects the sensory information to the pallium of the forebrain (green). (adapted from Derjean et al. 2010)

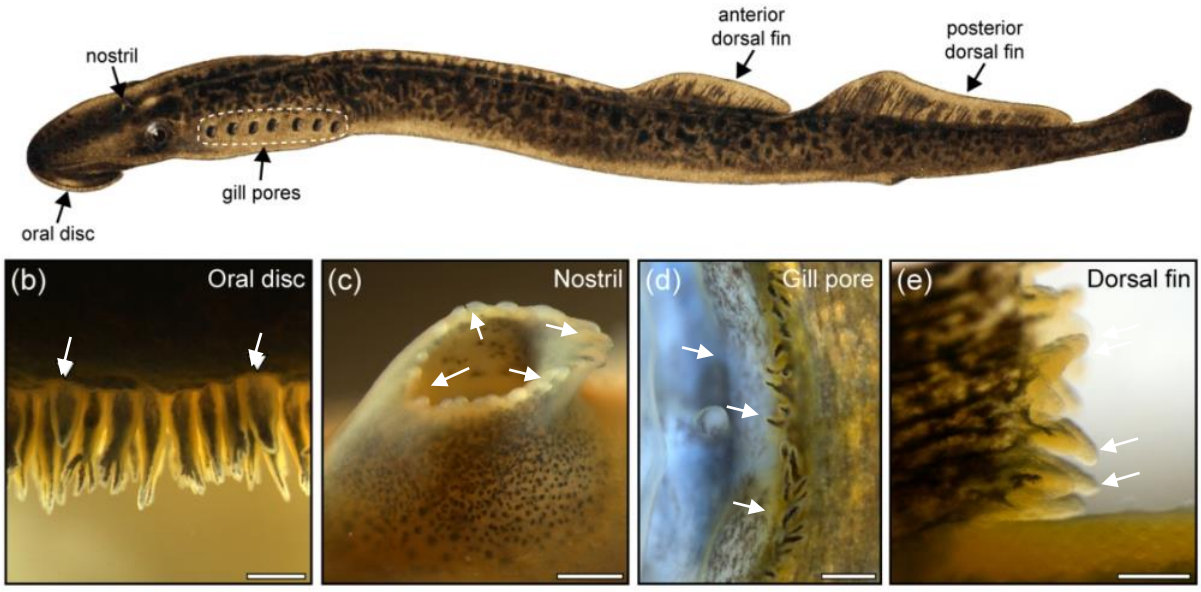

Figure 1.4: The location of papillae in adult sea lamprey

A visual representation of papillae (arrow) located on the nostril, oral disc, gill pores and dorsal tail fins of the sea lamprey. (adapted from Daghfous et al., 2019)

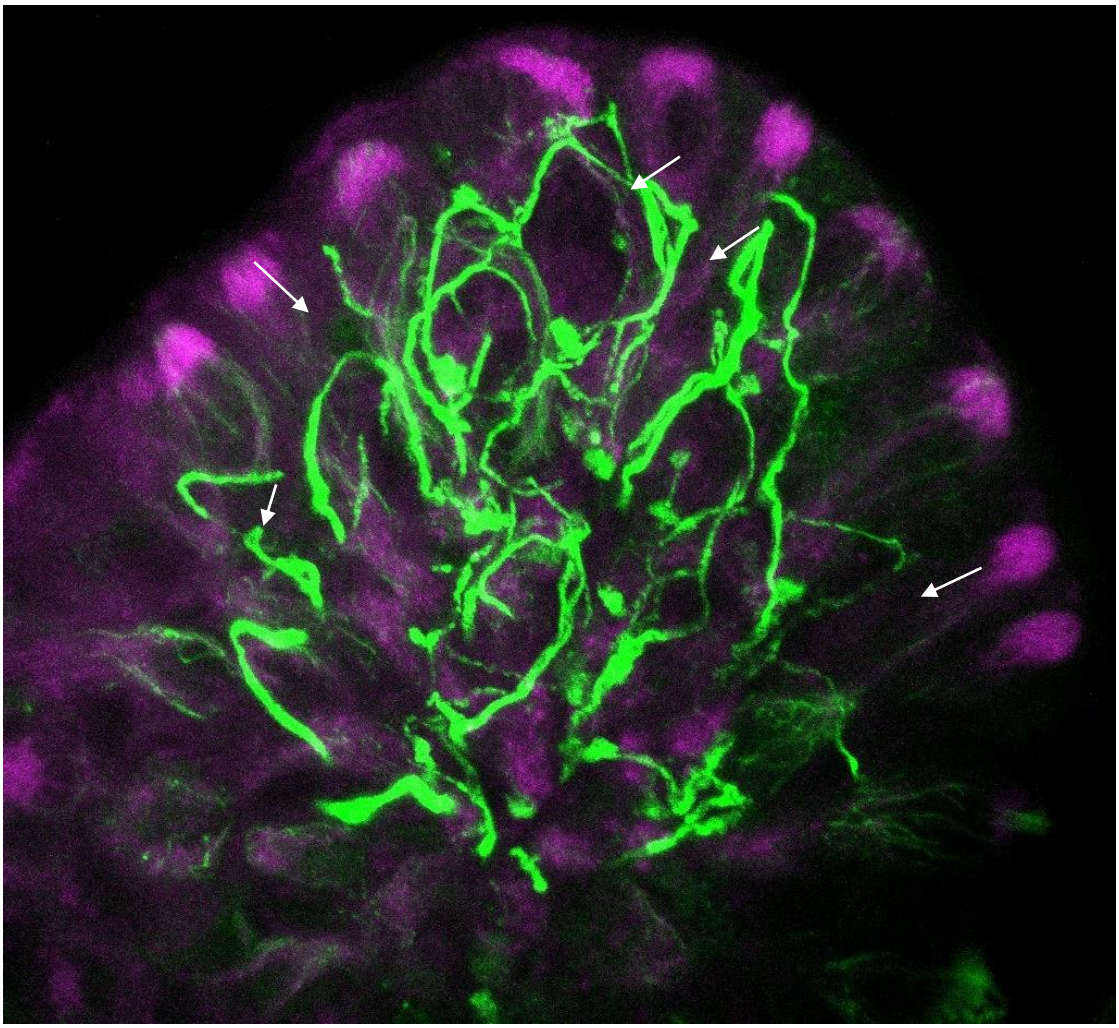

Figure 1.5: The immunolabelling of specialized cells, solitary chemosensory cells with calretinin and acetylated tubulin

The calretinin antibody has positively labelled “SSCs” around the peripheral of the olfactory epithelium while acetylated tubulin positively labels microtubules green. The arrows are indicative of the nerve fibres at the base of SCCs. This micrograph is a composite of 10 z-stack images taken on a confocal microscope. (adapted from Suntres, 2016)

Chapter 2: Olfactory sensory neuron responses to the polyamines, spermine and spermidine in the sea lamprey

2.1 Introduction

Aqueous organisms are surrounded by complex mixtures of chemicals, such as amino acids, sex steroids, and bile acid in which the olfactory system plays an essential role in detecting these chemical odorants. Olfaction commences with the binding of an odorant to receptors found on OSNs within the nostril (Munger et al., 2009). Each OSN expresses a single type of olfactory receptor (Mombaerts, 2004) which are members of a large seven-transmembrane multigene family, the G-coupled receptor family (Buck & Axel, 1991). It was found that the variability in the ligand-binding region of these seven-transmembrane proteins may provide a platform for these receptors to detect one or a mixture of odorants. Upon detection of the odorants the OSNs sends information to specific glomerular regions of the olfactory bulb and then onto higher brain regions (Munger et al., 2009). The question is how the OSNs, recognize these odorants and use them to encode odour identities. This may provide knowledge about how sensory information is processed in the brain and transformed into odorant perceptions, offering a "mechanism to accommodate the diversity of odour perception," as suggested by Buck & Axel, (1991). It has been discovered in mice (Nara et al., 2009; Saraiva et al., 2010) and honeybees (Carcaud et al., 2015) that most of the olfactory receptors have broad recognition abilities, meaning the olfactory receptor is recognizing more than one odorant. Some olfactory receptors are narrowly tuned, but they are able to recognize two different odorants with similar structures or functional groups which can be visualized using calcium imaging or patch-clamp recordings (Li et al., 2012; Carcaud et al., 2015; Zhang et al., 2013; Nara et al., 2009; Kida et al., 2018). The segregation of odorant signals is further separated into separate glomerular regions within the olfactory bulb and once that information is processed, it relayed to higher brain structures, where a specific

behaviour information is initiated, necessary for the fitness or survival of species (Johnson et al., 1998; Johnson & Leon, 2007; Friedrich & Korsching 1997; Munger et al., 2009).

From various studies, it has been discovered that amino acids, pheromones, and taurocholic acid has been identified as behaviorally relevant cues for sea lamprey and driving factors for many behaviours ranging from feeding to other social and reproductive behavioural cues (Li et al., 1995, 2002; Li & Sorensen, 1997; Siefkes & Li, 2004; Sorensen et al., 2005; Johnson et al., 2009, 2005; Siefkes et al., 2003). The effect of polyamines (spermine and spermidine) on the olfactory system and behavioural output is still unknown in the sea lamprey. Polyamines are present in all cells and possibly play a role in many mechanisms related to growth, degradation, and modulating ion channel activities (Tabor & Tabor, 1985; Morgan, 1999; Pavine et al., 2011). The linear aliphatic molecules and positively charged amino group of the polyamines gives them very similar structures to other potent aquatic odorants such as amino acids and biogenic amines (Tabor & Tabor, 1985).

There are many different types of polyamines such as cadaverine, putrescine, spermine and spermidine. Cadaverine and putrescine mostly induce repulsive behaviour in human and zebrafish because of its association with decay flesh and decay fish extract (Mietz et al., 1978; Ferrero et al., 2011; Hussain et al., 2013), whereas, not much is known about polyamine, spermine and spermidine. In plant, spermine and spermidine involved in facilitating growth and evoking stress response. Polyamine, spermine and spermidine were first discovered in human semen and play a role in sperm development and increasing sperm mobility in humans (Mann et al., 1974; Rosenthal et al., 1954; Shohat et al., 1990; Porat & Clark, 1990). They have been linked to facilitating fertilization in rats and human as they are both present in the ejaculate of humans, and mice (Stanger et al., 1982; Pavine et al., 2012; Tabor & Tabor, 1985). Previous

studies have indicated that semen plays a role in activating series of reproduction behaviour in some species (Wyatt et al., 2014; Perry et al., 2014; Carolsfeld et al., 1997). Semen acts as an "anti-aphrodisiac" to prevent other males from copulating with females or act as an "aphrodisiac" to increase mating behaviour. The male bitterling fish (*Rhodeus ocellatus*), releases semen in their surrounding environment resulting in an increasing egg deposition by ovulating females (Smith et al., 2018). In snake, airborne sex pheromones and substrates do deter courtships between ovulating females and other males (Shine et al., 2012). The pacific herring releases semen to surrounding environment to elicit serious of reproductive behaviour along with elevated steroid hormone during spawning (Carolsfeld et al., 1997). Thus, the compounds found in semen may play role in inducing reproduction related behaviour.

Spermine has been found in milt of sea lamprey and spermidine is precursor of spermine, thus; both polyamines may play role inducing reproduction related behaviour in sexually mature male and female lamprey. During mating season, a single male sea lamprey attracts multiple ovulating females when odorants are released to signal sperm availability for reproduction (Siefkes et al., 2005; Johnson et al., 2015). An observational study done by Johnson et al., (2015) stated that the bile acid pheromone 3kPZS plays a role in luring sea lamprey from far distances upstream to the nesting area where there may be another unidentified component near the nest to induce attraction from ovulating females. Polyamines may be another essential odorant in semen (milt) for sea lamprey to detect during reproduction. Scott et al., (2019) has reported that spermine acts as aphrodisiac in sea lamprey but does not note whether spermidine also contains the same properties of acting as an aphrodisiac. Previous spermine and spermidine response study have been only been conducted on a single receptor expressed in a mammalian system leaving the exploration of responses in an intact olfactory epithelium of the sea lamprey

unknown territory (Scott et al., 2019). Since spermine acts as an aphrodisiac and spermidine has a very similar structure, these two odorants may play a role in attracting females during the ovulation period in sea lamprey. Thus, both polyamines may bind to same receptor.

In this thesis, calcium imaging of single cells in undamaged live olfactory epithelium was used, which provides the ability to identify whether individual cells are responding to a specific odorant. For example, Grande, (2017) determined that the putative pheromone component 3kPZS which stimulates directional movement and foraging behaviour in female sea lamprey (Li et al., 2002; Siefkes et al., 2003; Johnson et al., 2005, 2009) stimulates specific cells in the olfactory epithelium; similarly, the pheromone, PAMS-24, a territory marker (Brant, 2015), also stimulates specific sensory neurons. Grande, (2017) determined that each putative pheromone stimulates a particular olfactory sensory neuron in the sea lamprey and based on these findings I hypothesize that each olfactory sensory cell in the main olfactory epithelium respond to specific odorants. This study will investigate whether spermine and spermidine odorants have separate receptor sites in comparison to amino acids, bile acids and each other while also observing if the "combinatorial code" changes in sea lamprey if concentration changes from higher to low concentration of spermine and spermidine.

2.2 Methods

2.2.1 Animal collection

All procedures were done according to guidelines provided by the Animal Care Committee of the University of Windsor and the Canadian Council on Animal Care. Larva-phase sea lampreys were collected from Hammond Bay Biological Station, Millersburg, MI and post-metamorphic stage (transformer-phase) sea lampreys were supplied by ACME Lamprey

Company, Harrison, ME. All animals were housed at the University of Windsor in aquaria at 6°C ± 1°C in dechlorinated water under static renewal conditions unit.

2.2.2 Odorant test solutions

All pheromones were tested at a concentration of 10^{-7} M, including 3kPZS, PAMS-24, and PSZ. Taurocholic acid and amino acids (arginine and histidine) were tested a concentration of 10^{-4} M. The odorant spermine was originally tested at a concentration of 10^{-3} M and then gradually decreased to a concentration of 10^{-15} M. The odorant spermidine started similarly to spermine with an original starting concentration of 10^{-3} M and then gradually reduced to a concentration of 10^{-11} M.

2.2.3 Calcium imagining dye loading in the olfactory epithelium

Calcium imaging involves the measurement of fluorescent changes during odorant responses in the olfactory epithelium, the method for loading the calcium reporting dye (calcium green dextran crystal) was adopted from Grande (2017). Firstly, the larva-phase sea lamprey was anesthetized in 0.03% tricaine methanesulfonate (MS-222) (pH 7.4; Sigma-Aldrich, Oakville, ON, Canada). Upon becoming unresponsive, the larva was removed from the MS-222 solution and placed on a dry petri dish. The nasal pore was then dried using a Kimwipe and the calcium green-1 dextran crystals (C6765, Potassium salt, 3000 MW, anionic) were directly applied into the nasal cavity by the delicate insertion of a needle ensuring no damage was caused to the olfactory epithelium by working under a dissecting microscope. A 5-minute rest period was followed to allow the dye to dissolve within the nasal cavity, and during this rest period chilled dechlorinated water was gently poured on the gills and tail. After a 5-minute resting period, the individual was then returned to the holding aquaria for at least 48 hours for the dye to enter the olfactory sensory neurons in olfactory epithelium before dissecting and imaging. To further

improve the adopted techniques, we allowed changes in the methodology of loading. After a 5-minute rest period, the specimen was put into chilled dechlorinated water odorized with 3kPZS and taurocholic acid. An addition 5 minutes of rest were allotted before transferring the lamprey to the holding aquaria for a minimum for 48 hours before the lamprey was dissected and imaged.

2.2.4 Preparation of the olfactory neurons for calcium imaging

After an incubation period, the sea lamprey that has previously been loaded with calcium green dextran was euthanized (0.03% tricaine methanesulfonate, pH 7.4; Sigma-Aldrich, Oakville, ON, Canada) and decapitated. Following decapitation, the head region was immediately submerged in ice-chilled and oxygenated Ringer's solution (130 mM NaCl, 2.1 mM KCL, 2.6 mM $CaCl_2$, 1.8 mM $MgCl_2$, 4 mM HEPES, 4 mM dextrose, 1 mM NaHCO3; pH 7.4) which was continually replaced every 5 to 10 min during dissection. The skin and surrounding tissue around the olfactory epithelium and nasal cavity were removed and the central region of the nasal cavity, containing the olfactory epithelium, was carefully excised from the decapitated head and gently mounted on a custom-built perfusion with the epithelium orientated to face the inflow of Fresh Ringer's solution and the odorant solution without disturbing the OSNs. The epithelium was continuously perfused with cold fresh oxygenated Ringers solution and odorants were applied into the perfusate at the appropriate experimental times.

2.2.5 Calcium imaging acquisition

A Nikon Eclipse 800 epifluorescence microscope equipped with 40X water immersion objective containing a fluorescein isothiocyanate excitation/emission filter set was used to record calcium responses of the olfactory sensory neurons loaded with calcium green dextran (fluorophore excitation/emission was 506/531 nm) (Figure 2.1). The fluorophore emitted light which was captured with an intensified CCD video camera (Photometrics CoolSNAP HQ; Roper

Scientific, Tucson, AZ) or Leica Camera (Leica DFC9000 GT, Leica Microsystems CMS GmbH, United Kingdom) and recorded (~1. 6 frames per s), using Metafluor imaging software (Molecular Devices, Sunnyvale, CA) (Figure 2.2). ImageJ and MATLAB software (the United States, The MathWorks Inc.) were utilized to quantitatively characterize the Ca^{2+} responses for comparison. These responses were stated as relative changes in fluorescence ΔF/F% and areas (ΔF/F•s) and compared between cells and stimuli (Viana Di Prisco et al., 2004; Derjean et al., 2010). Larvae and transformers were used because of the larger imaging field of view provided displaying more cells in comparison to spawning lamprey, thus, increasing the probability of detecting fluorescing OSN within the field of view (VanDenBossche et al., 1995).

2.3 Results

Calcium imaging experiments take advantage of a calcium influx in the olfactory sensory neurons which occurs in the presence of odorants. *In vivo* methods were tested to observe whether the OSNs within the main olfactory epithelium were successfully loaded with fixable fluorescent green dextran. It was observed that many cells were indeed loaded with dextran in tissues that were sectioned containing the main olfactory epithelium from the application of the *in vivo* method of the larval phase sea lamprey (Figure 2.2a). The loaded OSNs were identified by their morphologically identifiable elongated shape, enlarged cell body, and the presence of axon extending towards the base of the main olfactory epithelium (Figure 2.2b).

The response characteristics of the cells were determined when the odorants were presented to the larval olfactory epithelium previously loaded with calcium green dextran and prepared as a semi-intact preparation. The odorant tests included individual polyamines, amino acids, bile acids, as well as mixtures of two or three different odorants. Changes in the fluorescence emissions in response to the odorant's application was recorded; an increase in the

fluorescence intensity was illustrated by a sharp change from blue band to an orange/red band, and then back to the pre-response colour of blue (Figure 2.3). The abrupt change in colouring on the colormap along with the calcium spikes are indicative of a calcium influx occurring upon odorant-binding in the cells (Figure 2.3). The larval epithelial preparation was tested to determine whether it was Ca^{+2} ions that were the cause of change in the fluorescence intensity (Figure 2.3). The neutral amino acid, L-serine is not an odorant to the sea lamprey, because of this property it was used as a negative control in the experiments (Li, 1994; Figure 2.6, Figure 2.7, Table 1) to visually show a failed response on the larval epithelial preparation during testing (Figure 2.6, Figure 2.7, Table 1). When a concentration of 10^{-7} M of 3kPZS (a reproductive pheromone and a positive control odorant) was applied the cells produced a response (Grande, 2017; Li et al., 2013), similar response was also observed when the test odorant, spermine was applied at a concentration of 10^{-3} M. Each odorant was used individually (Figure 2.3), any unresponsive cells did not show the presence of any spikes; however, the red band in the colormap was observed in the individual odorant application of 3kPZS and spermine (Figure 2.3). During testing, the fluorescence emission of unresponsive cells remained at the baseline and the presence of response spikes and red band only appeared when cells encountered 3kPZS and spermine.

The temporary rise in intracellular Ca^{+2} ions in the OSNs is caused by the opening of calcium ion channels allowing Ca^{+2} ions to enter (Restrepo et al., 1993; Bozza et al., 1998; Leinders-Zufall et al., 2000). To test if the measured fluorescence signal was due to the opening of the Ca^{+2} ion channel during the binding of an odorant to the receptor, the concentration of Ca^{+2} ions was reduced in the Ringers solution to 1.0 µM from 2.6 mM by adding EGTA in Ringer's solution and the odorant solution, a strategy previously used by Leinders-Zufall et al.,

(1997). EGTA chelate Ca^{+2} ion from the solution by 1 to 1 ratio. The removal of extracellular Ca^{+2} eliminated the odorant response to 10^{-9} M spermine (Figure 2.4a, & b); upon the re-introduction of the Ca^{+2} ions into the Ringers solution, the odorant response returned to 10^{-9} M spermine (Figure 2.4c).

In this study 27 larval epithelial underwent calcium imaging experiments, and a total of 188 cells displayed an increase in fluorescence intensity (Figure 2.5, Figure 2.6, Figure 2.7). Among the 27 epithelial preps, 9 animals were tested with pheromones, taurocholic acid (TCA) and amino acids (Figure 2.5), 9 animals were tested with spermine, pheromone (3kPZS, PAMS-24), TCA, (Figure 2.6) and amino acids (arginine, histidine) and the last 8 animals were subjected to spermine, spermidine, TCA, and amino acids (Figure 2.7). Each test had odorants applied individually and in mixtures; all 27 preps showed that separate cells respond to specific odorants. In addition, spermine and spermidine were tested at multiple different concentration ranging from a potent concentration of 10^{-3} M to a diluted concentration of 10^{-15} M (Table 1). The larval preps were capable of sensing both spermine and spermidine at very low concentration (Table1).

Spermine and spermidine are odorant molecules with similar structure (Figure 2.1) having a similar carbon backbone and amine groups. The cells in the main olfactory epithelium of the larval phase sea lamprey were observed fluorescing to individual odorant components (i.e. spermine or spermidine) when tested individually and in the mixture (spermine + spermidine) (Figure 2.8, Figure 2.9). One of larval epithelial prep showed that 11 cells responded to spermidine and 12 cells responded to spermine (Figure 2.9) and none of the 23 cells responded to both spermine and spermidine at the same time. Also, both figures 2.8 and 2.9 show that the

separate cells respond to both odorants, even at high concentrations of 10^{-5} M spermine and spermidine.

Odorant testing was also completed with spermine and spermidine in a mixture with TCA (a bile acid). TCA is a potent odorant to sea lamprey (Li et al., 1995; Green et al., 2017), it is structurally cyclic and has a single amine group whereas, spermine and spermidine are linear aliphatic molecule with multiple amine groups (Figure 2.1). It was observed that the separate cells respond to TCA, spermine and spermidine (Figure 2.11, Figure 2.7). 11 different animal preps showed that none of the cells responded to more than one odorant (Figure 2.5, Figure 2.6, Figure 2.7). 10^{-4} M TCA was tested with a concentration of 10^{-3} M spermine which resulted in cells that responded to TCA but no overlapping with the cells that responded to spermine (Figure 2.10). This test once again showed that spermine and spermidine bind to separate cells at a concentration of 10^{-9} M. With the reduction in the concentration of spermine and spermidine, the specificity of the cells within the main olfactory epithelium did not lose their integrity (Figure 2.9). Both spermine and spermidine binding to separate cell at potent and diluted concentrations (Figure 2.8, Figure 2.9).

The amino acid, arginine is also potent amino acid for sea lamprey (Li & Sorensen, 1992), it is similar to spermine and spermidine as it also contains an amine group and carbon backbone (Figure 2.1). It was observed that arginine, spermine, and spermidine stimulated separate olfactory epithelial cells (Figure 2.12). When arginine, spermine, and spermidine were applied within a mixture, 3 cells responded. When these odorants were applied individually, the 3 cells fluoresced explicitly to specific odorants (Figure 2.12). Cell that responded to the concentration 10^{-4} M arginine (green) did not overlapped with the cells that responded to the concentration of 10^{-3} M of spermine (yellow) (Figure 2.13). The area colourmap micrograph

displays where increases in fluorescence intensity occurred (cell lit up) indicating that the odorant has bind to the receptor (Figure 2.14). The larval epithelium prep was tested with 10^{-4} M of arginine and 10^{-11} M spermine which is more diluted concentration than the 10^{-3} M spermine concentration that was previously used (Figure 1.14). Both tests (Figure 2.13, Figure 2.14) showed that the cells did not lose their specificity to the odorants (spermine and arginine), at both strong and dilute concentrations of spermine. Overall, the responses of each cell indicate that the individual odorants stimulate specific cells (Figure 2.8, Figure 2.11, Figure 2.12); TCA, amino acids and pheromones stimulate specific OSNs independently of the polyamine responding cells (Figure 2.15).

2.4 Discussion

This study shows that spermine and spermidine can stimulate OSNs in the main olfactory epithelium of larval sea lamprey coinciding with previous EOG records in the epithelium of zebrafish and goldfish indicated that these compounds do stimulate OSNs (Rolen et al., 2003; Michel et al., 2003). The study done by Scott et al., (2019), exhibited that spermine acts as an attractant to lure ovulating females toward the male's nest (Scott et al., 2019). In mice, both spermine and spermidine were tested to attract mice toward location, (Saraiva et al., 2010), the mice were attracted to spermidine and showed a neutral response to spermine. This study adds to the current investigation of spermine as are productive pheromone of sea lamprey and spermidine may elicit response related to reproduction or stress, seen in mammal and plant (Tabor & Tabor, 1984; Takahashi et al., 2010).

This study sought to investigate how sea lamprey process odorants in mixtures compared to individual odorants. The investigation of the odorant-response profile in sea lamprey provides vital insight about the cellular responses of pheromone components (3kPZS and PAMS-24),

amino acids (histidine and arginine), polyamines (spermine and spermidine) and taurocholic acid being detected by separate cells of the olfactory epithelium. No overlap in a single cell occurred in responds to multiple odorants. Each pheromone, polyamine and amino acid may be involved in eliciting a specific behaviour related to reproduction, food-seeking, and predator avoidance. In the sea lamprey, it has been observed that DKPES failed to elicit a behavioural response; however, the blend of DKPES and 3kPZS attracted a significant number of ovulating females into the baited nest (Li et al., 2013). Similarly, a study on *Drosophila* indicated that pure cis-vaccenyl acetate may have a role in courtship and mating but did not induce a behavioural response alone. When cis vaccenyl acetate was mixed with geranyl acetate the mixture was more attractive to flies than as a single odorant (Schlief & Wilson, 2007). The combination of different odorants may induce synergistic effects in sea lamprey. In other words, the blend of spermine and spermidine with other sex pheromones may increase the chances of luring the ovulating female to the baited nest.

This study indicates that the olfactory epithelial cells might be highly specific in responding to prospective ligands. This observation raised the possibility that the olfactory sensory cells in sea lamprey may have "specialist" cells that may act to trigger specific innate behavioural responses to key ligands. The combination of different odorants in a mixture may help determine behavioural responses in sea lamprey. Saravia et al., (2016) indicated that a mixture of attractive and aversive odorants did create a change to behavioural responses rather than eliciting a one odorant-specific behaviour. For example, Trimethylthiazoline, a predator odour usually inducing a fear response in mice was overruled by the presence of rose oil and woody odorants (Matsukawa et al., 2011; Murakami et al., 2012). The ability to have control over behavioural responses will help serve as a tool in controlling the population of sea lamprey

in the Great Lakes, where their population has exponentially expanded since their invasion (Fetterolf, 1980). This study opens the possibility to explore developing a way to deter ovulating females from mating males using a blend of different odorants with the goal of preventing breeding.

Across several separate olfactory epithelium preparations, the response of a single odorant to a single OSNs remained consistent. Polyamines, amino acids and bile acids were able to fluoresce specific cells when applied individually or in a mixture; however, the majority of these cells were narrowly tuned to single chemical compounds. It has been observed in other species, that both the "broadly" and "narrowly" tune blend of olfactory receptors (OR) and trace amino-associated receptors are present in the olfactory system (You et al., 2016; Zhang et al., 2013; Saravia et al., 2016; Nara et al., 2011; Schlief & Wilson, 2007). In the mammalian system, the MOR256-17 receptor responded strongly to the odorants, amyl methyl sulphide, bromobenzene, piperonal, and methyl hexanoate while having a modest response to isophorone and 2-isobutyl-3-methoxypyrazine. The receptor is broadly tuned to different functional groups aldehyde, alcohol, ether, ester, ketone, sulphide, and halogen. In the same study, it was found that another receptor, the MOR256- 22, acts as a narrowly tuned receptor, responding only to cinnamaldehyde and α, α-dimethyl benzenepropanol. Both compounds have similar structures, possessing a propyl group attached to a benzene ring with a terminal oxygen (Kepchia et al., 2017). Similarly, there are receptors found in humans that are highly specific to the odorants, 3-mercapto-2-methyl pentane-1-ol only binding to OR2M3 among 391 human ORs (Neo et al., 2017). In insects, one of DA1 ORNs responded exclusively to cis-vaccenyl acetate (Schlief & Wilson, 2007). The study that was conducted by Grande, (2017), and this research indicate that the olfactory sensory neurons are ultrasensitive to odorants. PAMS-24 and 3kPZS share a

tetracyclic (choline) backbone with sidechain branching off the carbon ring; spermine and spermidine share amine groups and a carbon chain backbone. Each of these compounds are binding to specific receptors found in sea lamprey (Libants et al., 2009). The receptor on OSNs within main olfactory epithelium are highly specific compared to the receptors found on OSNs within the mammalian system as they do not use combinatorial mechanism to detect structurally similar odorants.

The study indicates that both spermine and spermidine bind to specific cells, which was also supported by Scott et al., (2019), who reported that spermine binds to TAAR like receptor 348 while spermidine was unable to activate the TAAR-348 receptor. Spermidine may stimulate another TAAR- like receptors in sea lamprey. In mice, both spermine and spermidine bind to the TAAR-9 receptor because of their structural similarity (Sarivia et al., 2019). The OR and TAAR receptor gene of most evolutionary recent vertebrates may have diversified from ancestral vertebrate into many subfamilies, which may have resulted in the combinatorial coding scheme in mammals and teleosts. Lamprey may be showing high specificity to odorants because it is the earliest jawless vertebrate containing fewer OR and TAAR receptor genes. Understanding how lamprey process different odorants in mixtures and individually, provide an understanding of the evolution in vertebrate olfactory systems in terms of organization and function.

At lowers thresholds, the odorants bind to high-affinity sensory neurons and as the concentration increases fewer sensitive cells start recruiting, giving rise to the combinatorial coding scheme in mammals (Kaur et al., 1991; Mori et al., 1995; Hildebrand et al., 1997; Malnic et al., 1999); in other words, individual odorants start activating more than one receptor at higher concentrations. In sea lamprey, the olfactory sensory neurons did not lose specificity at higher or lower concentrations of spermine and spermidine. The study by Leinders-Zufall et al., (2000),

showed that the six structurally diverse ligands 2,5-dimethylpyrazine, 2-sec-butyl-4,5-dihydrothiazole, 2,3-dihydro-Exo-brevicomin, farnesene, 2-heptanone, and 6-hydroxy-6-methyl-3-heptanone, stimulate unique subsets of vomeronasal neurons in mammalian providing further support (Leinder-Zufall et al., 2000); these ligands are known to induce pheromonal activity in recipient female mice. In addition, the vomeronasal neurons did not respond to more than one of the six pheromones at any tested concentration. In zebrafish and goldfish, the cross-adaption experiment, which was done at a higher concentration of polyamines, also indicated that the polyamines have separate receptor sites than amino acid (Michel et al., 2003; Rolen et al., 2003). The olfactory system of sea lamprey may be unique since both pheromone and non-pheromones activate a non-overlapped subset of olfactory sensory neurons within the main olfactory epithelium. Perhaps the cells in the main olfactory epithelium are responsible for eliciting innate behaviour responses in sea lamprey.

This study also demonstrates that the main olfactory epithelium of larval lamprey can detect a very dilute concentration of 10^{-15} M spermine. Scott et al., (2019) had previously indicated that 10^{-14} M of spermine could stimulate the olfactory system of sea lamprey and attract ovulating females. Further, this study shows that spermidine could also be detected at high potency (about 10^{-9}M) by larva. Similarly, this high sensitivity has also been seen with other lamprey pheromones (Grande, 2017; Li et al., 2002; Siefkes et al., 2004). The molar detection threshold of PZS and PAMS-24 was found to be 10^{-12} M while PSDS was 10^{-13} M (Grande, 2017). The detection of sex pheromones at lower concentrations was also seen in other species such as goldfish (Stacey et al., 2015), tilapia (Kellar-Costa et al., 2014) and some insects (Gomez-Da et al., 2014). Overall, the amino acids, taurocholic acid, pheromones, and polyamines showed a one-cell-one-odorant response profile in the sea lamprey. The separate

cells for each odorant response profile may be an essential in stimulating a specific behavioural response. In the future, it would be beneficial to study whether all pheromones show the same specificity to OSNs at high concentrations as polyamines, spermine and spermidine. It has been observed in a mammalian system that not all cells have high specificity to odorants and as a result the neighbouring OSNs are recruited if the concentration of the odorant is increased (Kaur et al., 1991; Mori et al., 1995; Hildebrand et al., 1997; Malnic et al., 1999; Saraiva et al., 2010). This study only verified that the separate cell binds to polyamine, spermine and spermidine. No other polyamine, such as putrescine and cadaverine were tested that also have similar structure to spermine and spermidine. Thus, it would be good to verify that cell's specificity was seen with all polyamines for future studies. In zebrafish and goldfish, it has been indicated that the polyamines might not activate the cyclic AMP pathway and the IP_3 pathway (Rolen et al., 2003; Michel et al., 2003). In the sea lamprey the cells response to pheromones was reduced to baseline after the application of forskolin, an adenylate cyclic activator. This indicates that the cells detecting pheromones use the cyclic AMP pathway (Grande, 2017), making it essential to study the transduction pathway used by the cells detecting spermine and spermidine. Understanding of transduction pathway, helps to understand how the olfactory system work in sea lamprey. The ratio in a mixture of attractive odorants and aversive odorants may attribute to a simple mutual cancellation of attractive and aversive signals in the brain which needs to be further investigated as it helps determine how the population of sea lamprey can be managed in the Great Lakes.

2.5 References

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2.6 Figures

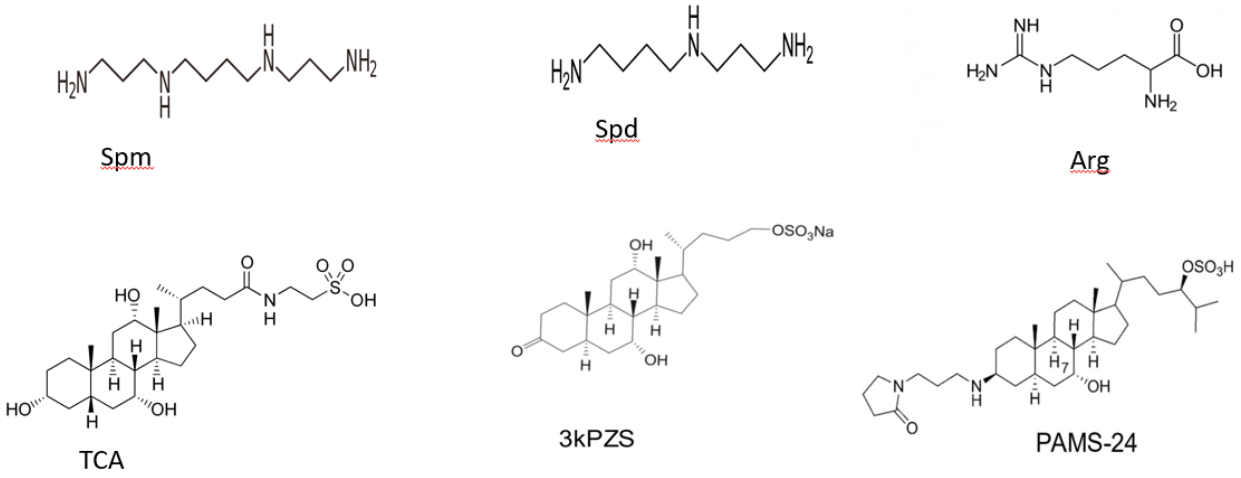

Figure 2.1: The chemical structure of the compounds known to elicit an olfactory response in sea lamprey

Spermine (Spm), Spermidine (Spd), Arginine (Arg), Taurocholic acid (TCA), 3-keto petromyzonal sulphate (3kPZS) and petromyzonamine-24-monosulfate (PAMS-24). Spm, Spd and Arg have similar carbon chain backbone and amine group. TCA, 3kPZS and PAMS-24 are bile acid, ring structure with carbon chain. (Li et al., 1995, 2012, 2013 ; Scott et al., 2019, Michel et al., 2003, Green, 2012).

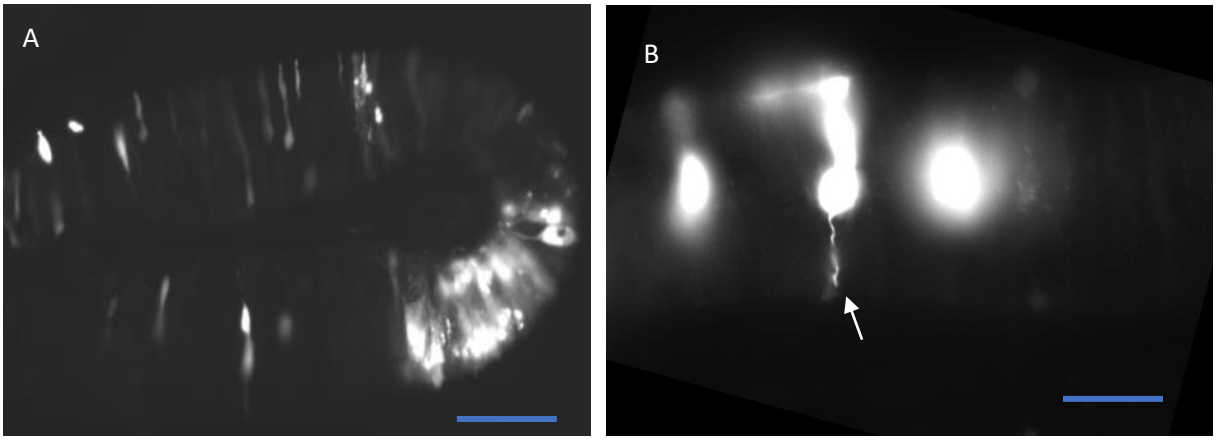

Figure 2.2: Olfactory epithelial cells labelled by anterograde loading through the nostril with fluorescent dextran

Fluorescent dextran was inserted into the nostril to label cells in the main olfactory epithelium. A) Visualization of olfactory epithelium cells being successfully labeled. B) An olfactory sensory neuron was identified by the columnar shape and the presence of an axon (arrow) extending from the basal surface of the cell. The scale bar in A) is 125 µm and B) is 60 µm.

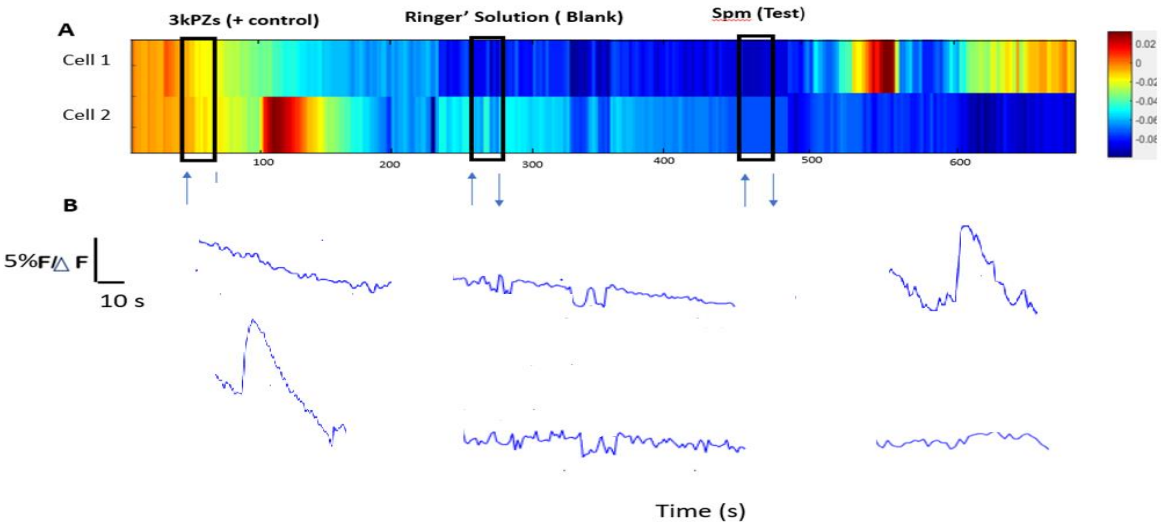

Figure 2.3: Calcium imaging showing responses to odorants 3-keto-petromyzonal-sulphate (3kPZS) and spermine (Spm)

An increase in fluorescence intensity was seen in Cell 1 following the application of Spm, but there was no response seen for 3kPZS or Ringer's solution. In the case of Cell 2, fluorescence intensity increased during the application of 3kPZS, but not the Ringer' solution (a negative control) or 10^{-3} M Spm, showing that separate olfactory epithelial cells are responding to separate odorants.

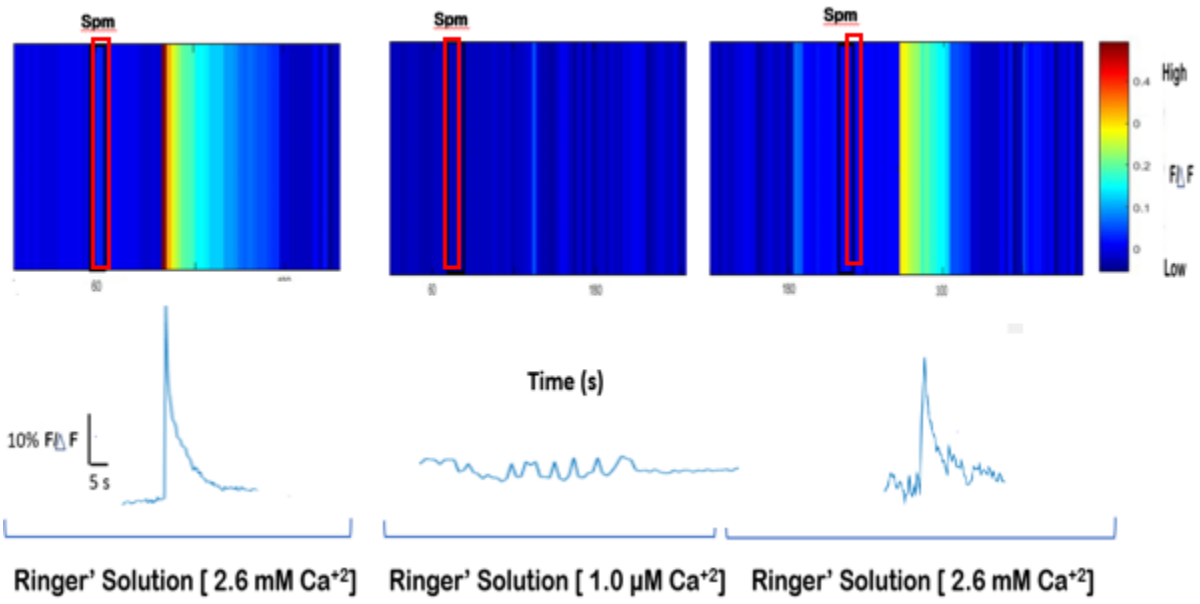

Figure 2.4: Odorant responses to spermine require extracellular calcium

1. A calcium spike was observed following the application of 10^{-9} M spermine when the epithelial prep was bathed in a Ringer's solution containing 2.6 mM $CaCl_2$. The blue spike is indicative of the cell response to spermine. The blue color in the color map indicates the baseline emission whereas the yellow or red indicates an increase in fluorescence emission denoting a response.
2. When the epithelial prep was bathed in Ringer's solution with less calcium (1.0 mM calcium), no change in the fluorescence emission was seen following the application of spermine.
3. Following the re-introduction of Ringers solution with 2.6mM calcium chloride, a florescence spike was seen following the application of spermine. The blue color on the color map represents the base line emission whereas the yellow or red signifies an increase in fluorescence emission indicating a response.

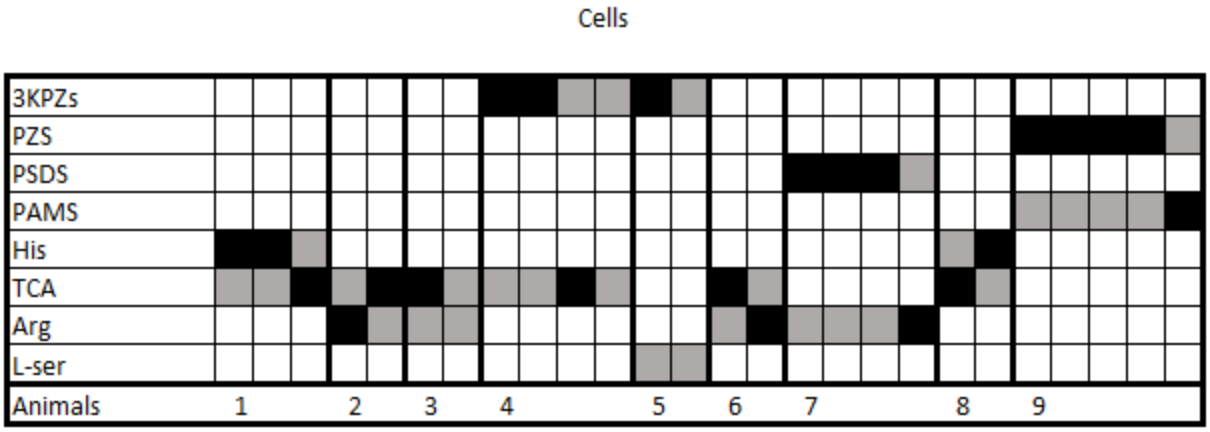

Figure 2.5: Separate olfactory epithelial cells respond to each odorant

Multiple odorants were tested on the main olfactory epithelium using the calcium imaging method. The columns represent and provide a visual representation of odorant responsive cells in olfactory epithelium. Each black square ■ show that the cell is responding to a specific odorant, the grey square ▭ indicates that the cell is showing no response to an odorant. The white square □ indicates that the odorant was not tested in an experiment. The thick black line separating groups of the columns specifies the different preps (9 different lampreys). The odorants that were tested included 10^{-7} M 3kPZS (3 ketopetromyzonol sulfate), 10^{-7} M PAMS (petromyzonaamine-24-monosulfate), 10^{-4} M Arg (arginine), 10^{-4} M TCA (taurocholic acid), and 10^{-4} M his (histidine).

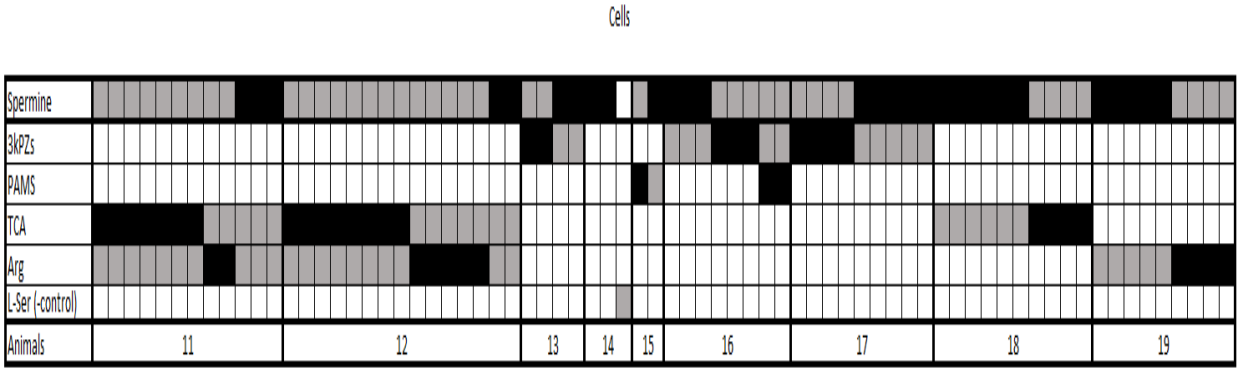

Figure 2.6: Separate olfactory epithelial cells responded to spermine and other odorants. Spermine was tested during 9 experiments along with different odorant individually and in mixture. 72 cells responded. Each test indicated that spermine bind to a specific receptor. The columns represent exhibiting an odorant response within the olfactory epithelium using calcium imagining. Each black square ■ shows that the cell is responding to a specific odorant, the grey square ▭ indicates that the cell is not responding to the odorant. The white square ▭ indicates that the odorant was not tested in an individual experiment. The thick black line separating groups of columns indicates the separate epithelial preps (9 different lamprey). The odorants tested include 10^{-7} M 3kPZS (3 ketopetromyzonol sulfate), 10^{-7} M PAMS (petromyzonaamine-24-monosulfate), 10^{-4} M Arg (arginine), 10^{-4} M TCA (taurocholic acid), and spermine with concentrations ranging from 10^{-5} M to 10^{-11} M.

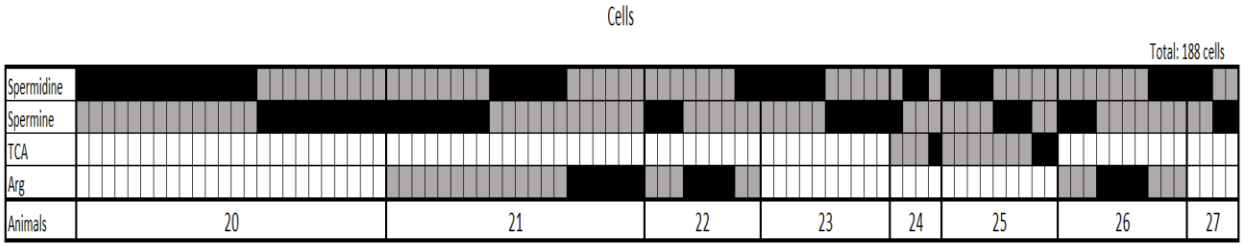

Figure 2.7: Separate olfactory epithelial cells responded to spermidine and other odorants

Spermidine was tested in 7 animals along with individual test involving other odorants. 87 cells were responsive. each test indicated that spermidine would bind to specific receptor different from spermine and the other odorants. The columns represent individual cells and their odorant responses observed using calcium imagining within the olfactory epithelium. Each black square ■ shows that the cell is responding to a specific odorant, the grey square ▭ indicates the cell did not respond to any odorant. The white square ▭ indicates that odorant ~~is~~ was not tested during any individual experiments. The thick black line separating groups of columns indicates the separate epithelial preps (9 different lamprey). The odorants tested included 10^{-4} M Arg (arginine), 10^{-4} M TCA (taurocholic acid), spermine with a range in concentration from 10^{-5} M to 10^{-11} M, and spermidine also including a range in concentration from 10^{-3} M to 10^{-11} M.

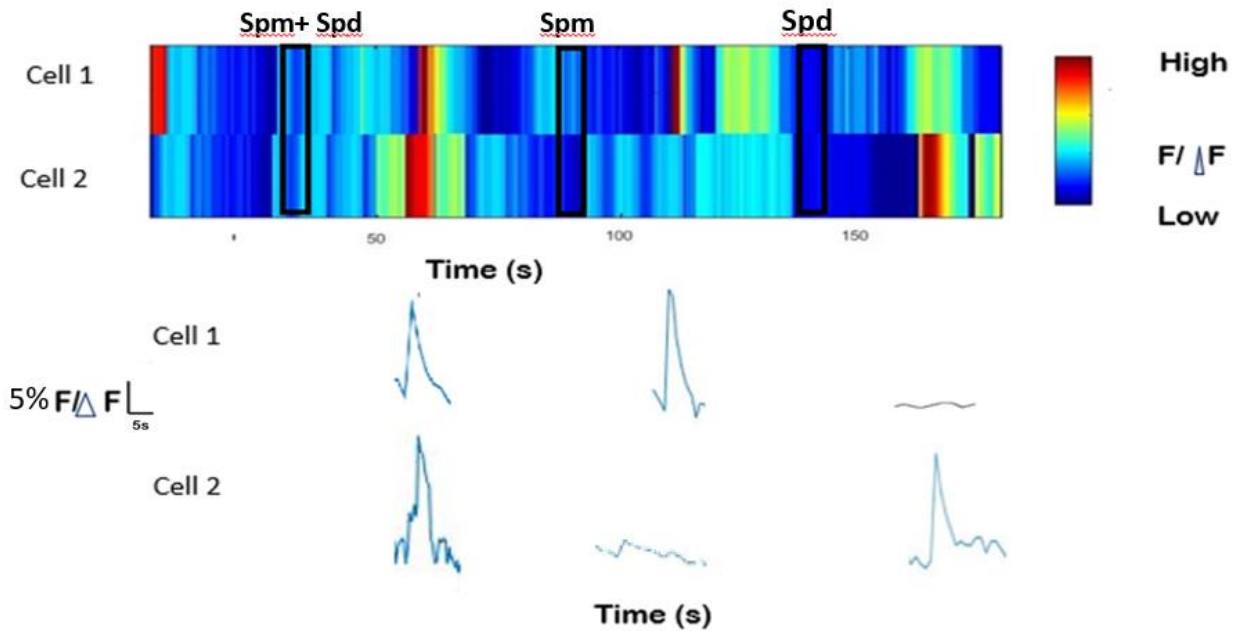

Figure 2.8: Separate olfactory epithelial cells respond to spermine and spermidine

Fluorescence emissions from two separate cells in larval stage sea lamprey were recorded from a mixture of 10^{-5}M Spm +10^{-5}M Spd, 10^{-5}M Spm, and 10^{-5}M Spd. The vertical red band illustrates the excitation response from cells when they encounter the odorants; the blue indicates the baseline emissions. The mixture and individual odorants were applied for 5 second (shown within the black box)

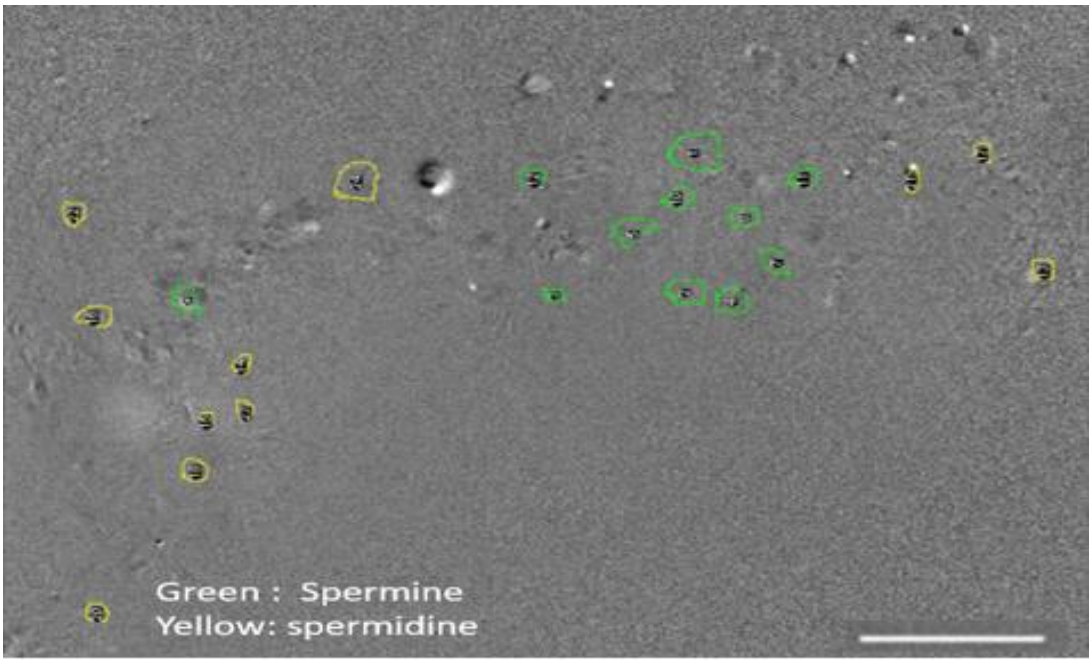

Figure 2.9: Location of responding cells to spermine and spermidine in larval olfactory epithelium

Superimposed trace images were generated from calcium imaging, the background was subtracted and divided by average delta F using ImageJ software. The traces show the location of cells that were responding during separate applications of each odorant; 10^{-5} M spermidine (yellow traces) and 10^{-5} M spermine (green traces) on the same olfactory epithelial prep. Scale bar is equal to 60 μm.

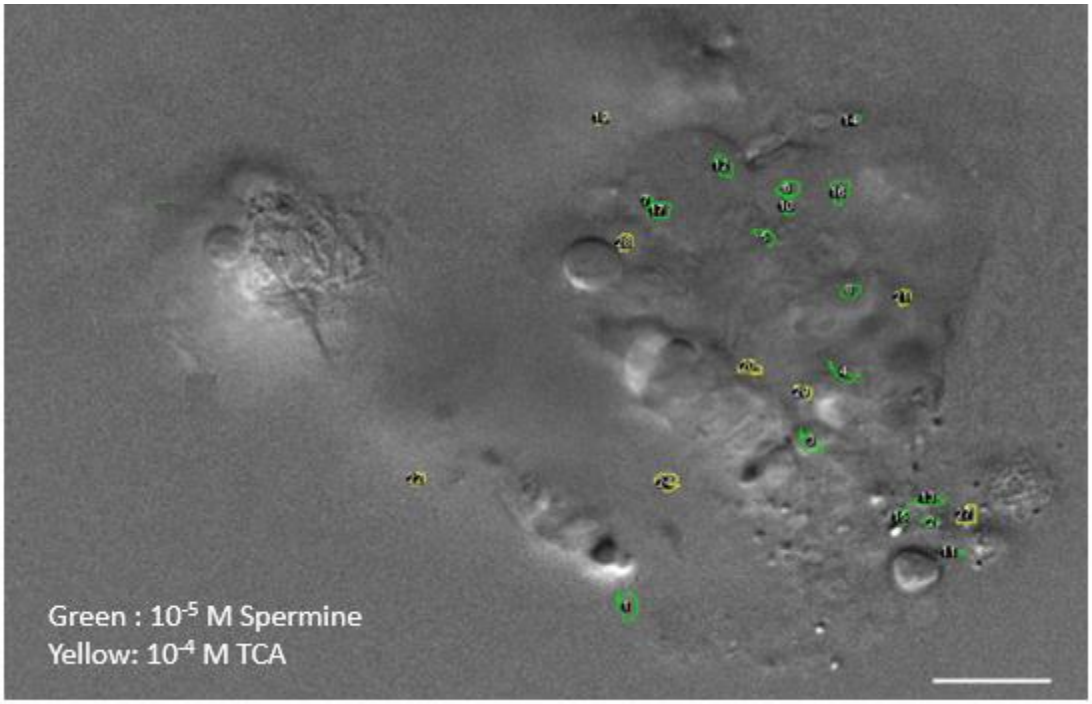

Figure 2.10: Location of larval olfactory epithelial cells in response to taurocholic acid (TCA) and spermine

Superimposed trace images were generated from calcium imaging using ImageJ software. The two sets of traces were taken from separate applications of each odorant; 10^{-4} M taurocholic acid (yellow traces) and 10^{-5} M spermine (green traces) onto the same olfactory epithelial prep. Scale bar represents 60 μm.

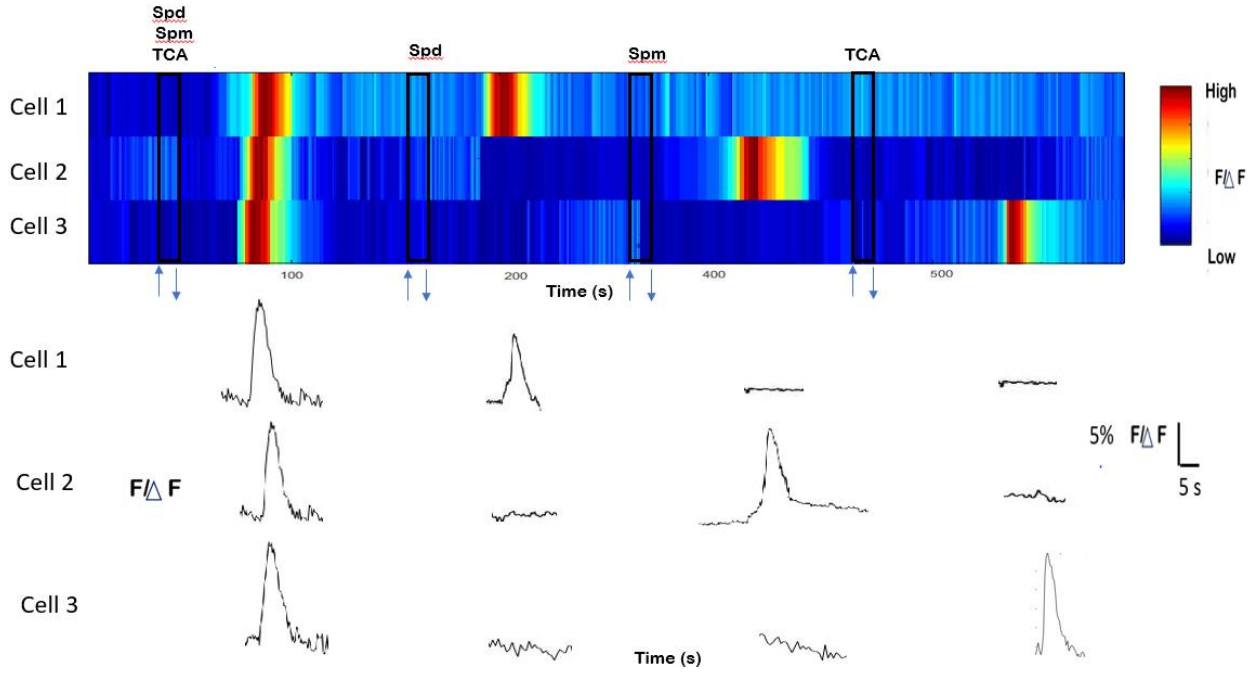

Figure 2.11: Separate cells respond to taurocholic acid (TCA), spermine (Spm) and spermidine (Spd)

Fluorescence emissions from three cells in larval sea lamprey that were subjected to a mixture of 10^{-9}M Spd+10^{-9}M Spm and10^{-4}M TCA, 10^{-9}M Spd, 10^{-9}M Spm, and 10^{-4}M TCA. The red vertical bands illustrate the stimulation of cells in response to the odorants; blue indicates the baseline emissions. The odorants were applied for 5 second (shown in the frame of the black box). The fluorescence intensity transients are shown below the color image.

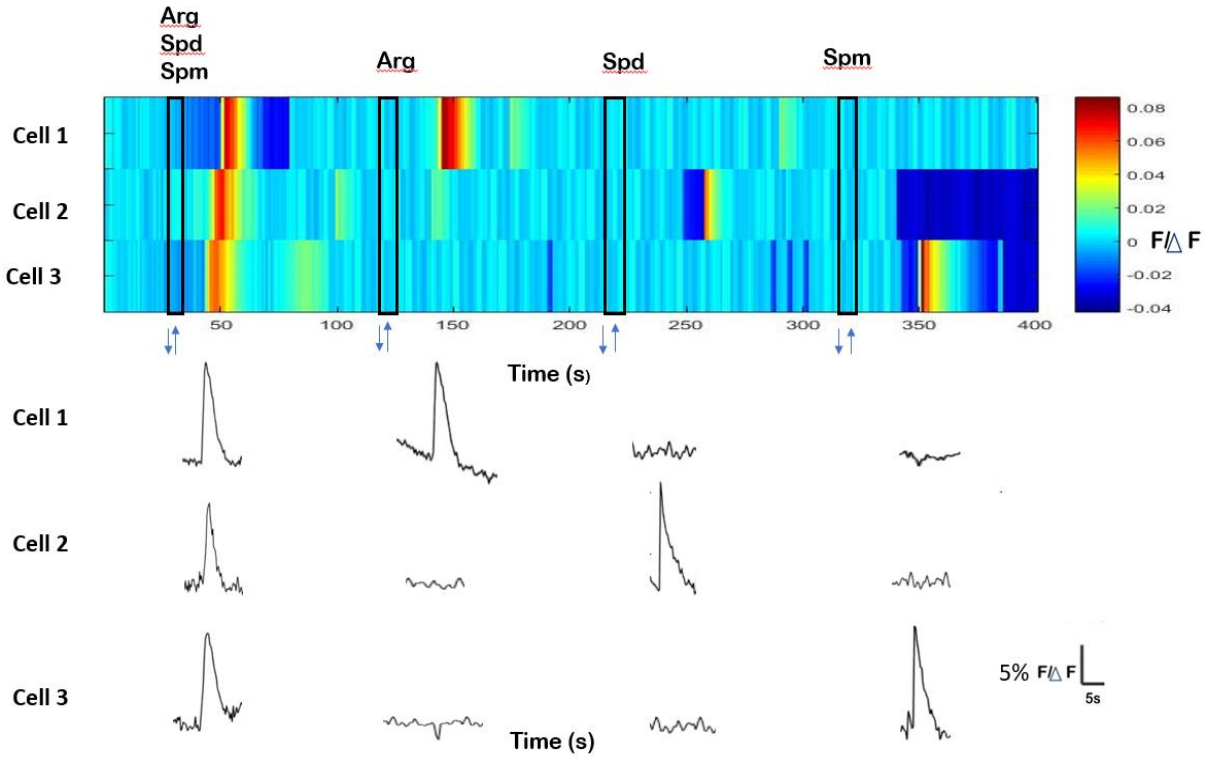

Figure 2.12: Separate olfactory epithelial cells respond to arginine, spermidine and spermine

Fluorescence emissions from three cells in larval sea lamprey during the application of the mixture containing 10^{-4}M Arg;10^{-9}M Spd; ^{9}M Spm, 10^{-4}M Arg, 10^{-9}M Spd and 10^{-9}M Spm were recorded. The red vertical band illustrates the change in fluorescent intensity from the blue baseline. The odorous stimulants were applied for 5 second (depicted in the frame of the black box). The fluorescence intensity transients are shown below the color mapping.

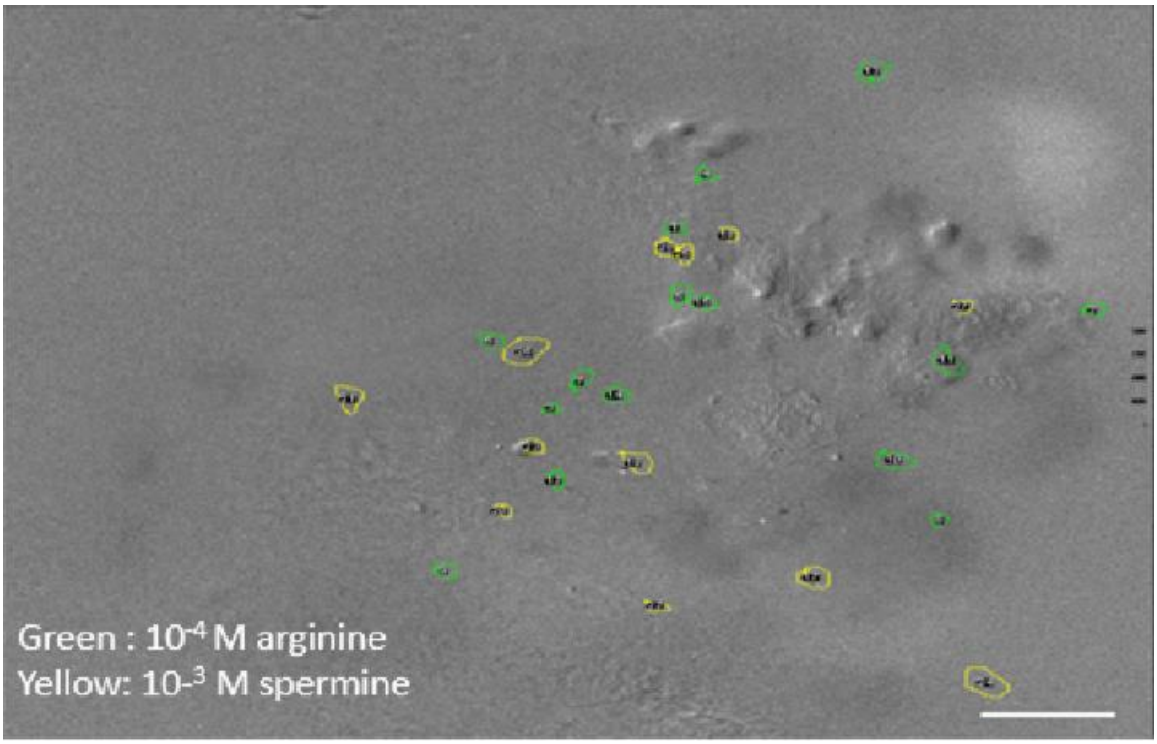

Figure 2.13: Location of larval olfactory epithelial cells responding to arginine and spermine

The superimposed traces were generated from calcium imaging and processed using ImageJ software. The two sets of traces were taken from separate applications of each odorant (10^{-3} M spermine (yellow traces) and 10^{-4} M arginine (green traces) onto the same olfactory epithelium. Scale bar equals 60 μm.

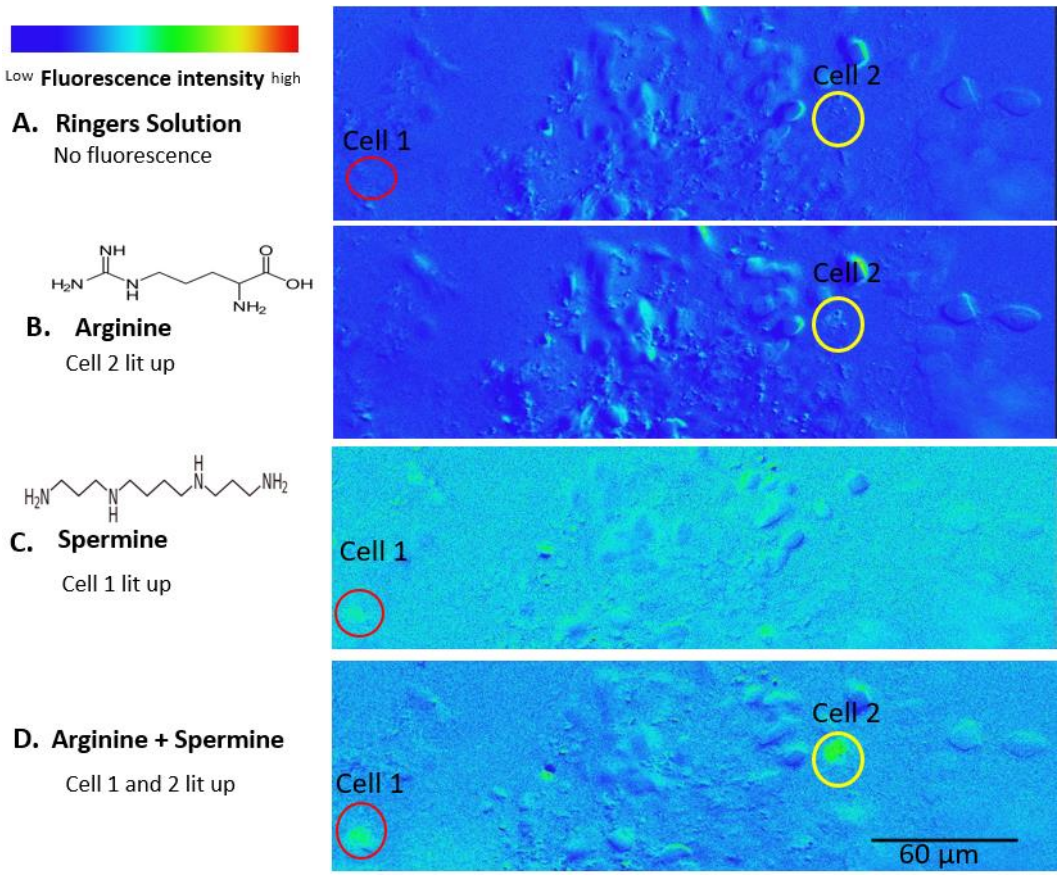

Figure 2.14: Separate olfactory epithelial cells respond to spermine and arginine

A heat contour map was applied to each image ranging from blue (no fluorescence) to red (highest fluorescence). Panels A-C depict the same two cells in the same field of view with two circles (yellow and red) indicating the fluorescing cells in each case. A) shows that two cells (yellow& red circle) did not respond when there was the absence of an odorant. B) shows cell 2 responds when 10^{-4} M arginine was applied (yellow circle), but cell 1 did not respond. C) shows cell responds when 10^{-11} M spermine was applied (red circle), whereas cell 2 did not respond. D) shows both cell 1and cell 2 (yellow & red circle) fluoresced when a mixture of both 10^{-4} M arginine and 10^{-11} M spermine was applied.

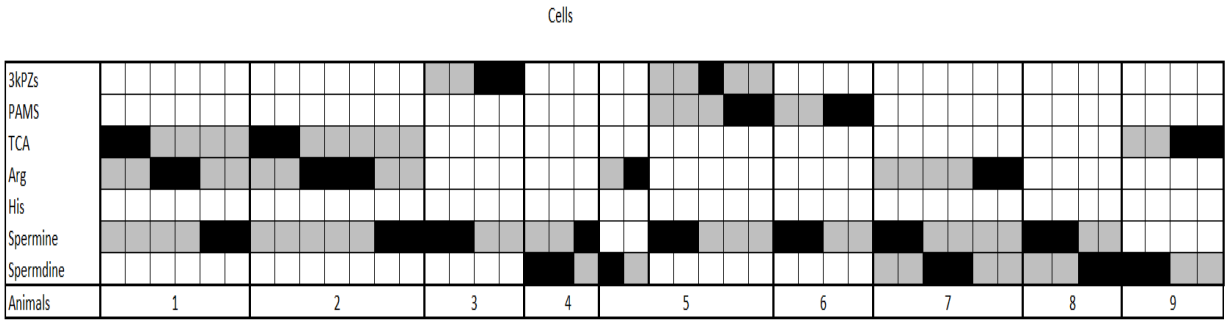

Figure 2.15: Separate olfactory epithelial cell responds to each odorant

This figure indicates that each cell is stimulated by a specific odorant. The columns are representative of cells and denote odorant responsiveness in the olfactory epithelium using calcium imagining. Each black square ■ shows that the cell is responding to a specific odorant, the grey square ▭ indicates the cell is not responding to odorants. The white square □ is indicativeof odorants not tested in an individual experiment. The thick black line separating groups within the column represents separate epithelial preps (9 different lamprey). The odorants tested include 10^{-7} M 3kPZS (3 ketopetromyzonol sulfate), 10^{-4} M Arg (arginine), 10^{-4} M TCA (taurocholic acid), spermine with a concentration range of 10^{-5} M to 10^{-13} M and spermidine with a concentration range of 10^{-3} M to 10^{-15} M.

Table 1: The number of animals tested for olfactory sensory response at different odorant concentrations (+) indicate the animals responded to the odorants, (-) there was no response

Odorant Tested	Concentration	Animal with responses	Animal with no response	% of animal response
Spermine	10^{-15} M	2+	3-	40
	10^{-13} M	5+	1-	83
	10^{-11} M	4+		100
	10^{-9} M	6+		100
	10^{-7} M	7+		100
	10^{-5} M	7+		100
	10^{-3}M	4+		100
Spermidine	10^{-11} M	2+	2-	50
	10^{-9} M	5+		100
	10^{-7} M	3+		100
	10^{-5} M	2+		100
	10^{-3} M	2+		100
Arg	10^{-4} M	9+		100
TCA	10^{-4} M	10+		100
L-Serine	10^{-4} M		3-	0
Forskolin	10^{-4} M	4+		100
3kPZS	10^{-7} M	6+		100
PAMS-24	10^{-7} M	4+		100
PZS	10^{-7} M	2+		100
PSDS	10^{-7} M	1+		

Chapter 3: The hardships of obtaining odorant responses in the accessory olfactory organ using calcium imaging in sea lamprey

3.1 Introduction

The survival of an organism depends on its ability to communicate and move within its environment. For fish, the chemosensory-mediated locomotion is essential for reproduction and survival; for example, sexually mature male salmon sense amino acids in the urine of ovulating females resulting in motor behaviour in the males (Moore & Scott, 1992). The male move toward the female to reproduce next generation. The olfactory system plays an important role in inducing locomotion in fishes (Daghfous et al., 2016; Johnsen & Teeter, 1985) and mammals (Porter et al., 2007). The sensory information is initially detected by receptors found on the dendrites of the olfactory sensory neurons. The signal travels through a series of neural relays in the central nervous system resulting in a logical motor behaviour (Munger et al., 2009; Derjean et al., 2010). Sexually mature sea lamprey travels long distance to find the nesting area by smelling migratory pheromones released by the larva (Johnson et al., 2009). Derjean et al., (2010) and Green et al., (2017) identified that the medial region of the olfactory bulb detects a variety of odorants which then activate reticulospinal cells; the motor control cells. Using retrograde labelling, the medial region involved in receiving the signal from the AOOs were also identified. Furthermore, the medial region is biochemically different from other areas of olfactory blub receiving input from the main olfactory epithelium.

However, it has yet to be discovered what is happening in real-time within the AOO. There has been no electrophysiological recording of odorant responses within the AOO of sea lamprey due to its difficult location as small pocket below the olfactory epithelium. The AOO was first discovered in (1887) by W. B. Scott, who concluded that this tissue is a diverticulum of the main olfactory epithelium and found in the caudoventral regions of the peripheral organ in lamprey. It appeared as an epithelial pocket surrounded by nerve fibres and blood vessels

(Hagelin & Johnels et al., 2010), similar to the vomeronasal organ in rats (Soler & Suburno, 1998). Both AOO and vomeronasal have a tubular structure connected via duct to outer environment. The cells within AOO are ciliated whereas vomeronasal organ are microvillus (Munger et al., 2009, Green, 2012). The histology and biocytin retrograde labelling of spawning lamprey showed that the cells in the AOO are round & short, and the olfactory knob is more flattened and less bulbous like the crypt cells or "cul-de-sac" region of mammals (Green et al., 2017; Gonzalez et al., 2010; Greer et al., 2016). The crypt cells of lungfish (Gonzalez et al., 2010) or the olfactory sensory cells found in the "cul de sac" region of mammals both respond to more than one odorant (Greer et al., 2016); however, it is not known whether the sensory cells in the AOO respond to one or multiple odorants.

Can we characterize the cells in the AOO and the odorant responses in individual sensory neurons within the AOO and its transducing pathways using a different method, such as calcium imaging? The calcium imaging technique has been used to identify odorant-responses in real-time on a single cell or intact tissue. This technique takes advantage of the calcium concentration change in the cells that occurs during the binding of odorants to cells. This approach is beneficial as it has the ability record the activity of many neurons in a fully intact brain simultaneously using fluorescent indicators which can be synthetic (Stosiek et al., 2003) or genetically engineered (Hasan et al., 2004; Chen et al., 2013). Fluo-4 AM (Weiss et al., 2018) monitors action potentials, which complements microelectrode recording (Smetters et al., 1999). The use of the calcium indicator (calcium green dextran), has been successful in monitoring the neuronal activity in intact olfactory epithelium of sea lamprey (Grande, 2017) and the motor cells (Derjean et al., 2010).

To determine whether the olfactory sensory neurons of the AOO respond to odorant stimulation in a specific manner, the method of dextran crystal loading into the sensory cells of the AOO needed to be determined. It was decided to use several dye loading strategies in four different life stages of sea lamprey; larvae, newly transformed, parasitic and adult. *In vivo* techniques with and without the application of the odorant and electroporation technique were used. During the *in v*ivo technique, the syringe was used to delicately put dextran crystals in the nostril where they were readily taken up by the olfactory cells. This dye can travel long distances and has a longer transport time (up to 14 mm and 4 days) as mentioned in McPherson et al., (1997). Throughout the electroporation method, the cells were exposed to an electric field of sufficient amplitude, resulting in increased permeability of the cell membranes (Tsong, 1991; Neumann et al., 1999; Napotnik & Miklavcic, 2018). This method was sufficient because it allowed the reversible permeabilization and a rapid resealing of the membrane, provided that the amplitude was not overtly high and the cells returned to their initial state after exposure (Napotnik & Miklavcic, 2018). The electroporation method was used to deliver DNA, RNA, protein, and fluorescent indicators (Hass et al., 2001; Inoune et al., 2001; Chen et al., 2017; Rathenberg et al., 2003). Chen et al., (2017) was able to load acetoxymethyl form Ca^{+2} dye in neurons at the dorsal root ganglion using the electroporation method, which was not possible by incubating the tissue in the dye-containing solution. In this study, the cells of the AOO were labelled with acetylated tubulin to identify its neuronal identities and its location concerning the main olfactory epithelium. Moreover, GSIβ4 lectin labelling was also used to visualize D-galactose residues in the peripheral organ of the sea lamprey (Zielinski et al., 2000; DeBray et al., 1981). In various vertebrates, the axolemma and terminal buttons of the small diameter primary sensory neurons contain these glycoconjugates (Gerke & Plaenderleith, 2002). These

glycoconjugates may play an important role in cell to cell recognition or adhesion during development (Lidierth & Wall, 1998).

3.2 Methods

3.2.1 Animal collection

All procedures were completed according to the guidelines provided by the Animal Care Committee of the University of Windsor and the Canadian Council on Animal Care. Larva(n=35), parasitic (n=4) and spawning (n=7) sea lamprey were collected from Hammond Bay Biological Station, Millersburg, MI; post-metamorphic (transformer-phase) lamprey were supplied by ACME Lamprey Company, Harrison, ME (n=16). All animals were housed at the University of Windsor in aquaria at 6°C ± 1 °C in dechlorinated water under static renewal conditions. A total of n=62 was used during the experiments.

3.2.2 GSIβ4 labelling of the AOO and main olfactory epithelium

GSIβ4 labels the axons of olfactory sensory neurons by binding onto the galactosyl residues present, found in the glomeruli of the olfactory bulb (in both the medial and non-medial region) (Green et al., 2013). It was utilized to show the spatial relationship between the AOO and the duct that connects the AOO with the main olfactory epithelium. For GSIβ4 labelling, the tissue was sectioned at 18-20 µm on Fisher Brand Super Frost plus slides using Leica CN3050 S cryostat. The sections were immersed into acetone at -20°C for 10 minutes followed by rehydration for 10-15 minutes in 0.1 M PBS. The sections were then incubated in biotinylated Griffonia simplicifolia lectin1 (GS-1 isolectin B4, Cat log # B-1205, Vector, Burlingame, CA; 10 µg/ml in 0.1 M PBS, pH 7.4) overnight at 4°C temperature on the shaker. Slides were then washed every 30 minutes twice while still remaining on the shaker at 4°C and followed by incubation in DCS avidin-fluorescein for 5 to 24 hours (1:100 in 0.1 M PBS, Cat log 201,

Vector, Burlingame, CA, pH 7.4). Slides underwent acylated tubulin labelling, then were washed with 0.1 M PBS and mounted with Vectasheild.

3.2.3 Labelling of the peripheral organ with acetylated tubulin

The tissue sections of peripheral organs were immunolabelled with acetylated tubulin allowing the visualization of cilia and microtubules. Green (2012) immunolabeled the cells of the main olfactory epithelium and AOO of spawning lamprey to verify its neuronal identities. Cryosections foregoing acylated tubulin labeling, were immersed in acetone at -20°C for 10 minutes followed by rehydration for 10 to15 minutes in 0.1 M PBS. Sections were then incubated in acylated tubulin antibody (1/1000 or 1/2000 in 0.1 M PBS plus 5% goat serum, sigma, pH 7.4) for two days at 4°C on a shaker. At the end of this period, slides were washed every 30 minutes for 3 cycles while remaining on the shaker at 4°C before being moved into Alexa 488 conjugated anti-mouse secondary antibody for 3 hours or overnight incubation (1: 250 in 0.1 M PBS; A21151 Invitrogen). After incubation, the tissues were washed with 0.1 M PBS and mounted with Vectasheild.

3.2.4 Solutions and dyes

Calcium green dextran was used to load the sensory neurons of the main olfactory epithelium and the AOO using two different molecular weight of the dye dextran Texas Red (D3328, 3000 MW, Lysine Fixable). Depending on the life cycle stage of the sea lamprey, the dye was mixed with 0.01% Triton-X plus 0.1M NaCl. In larva, crystal was mostly put without mixing dye with Trition-X plus 0.1 M NaCl whereas, dye was always mixed with Trition-X plus 0.1 M NaCl after metaphoric stage. The odorant solution used in this experiment were 10^{-3}M spermine, 10^{-3}M spermidine,10^{-7}M 3kPZS (pheromones), and 10^{-4}M TCA (bile acid) mixed in 100 ml of dichloride water or 10 ml 0.01% Triton-X plus 0.1M NaCl.

3.2.5 *In vivo* loading of dye into the main olfactory epithelium and AOO

Calcium imaging was used to obtain measurement of the fluorescent changes during the olfactory sensory neuron's response. To perform calcium imaging, the dextran must enter the olfactory sensory neurons of the AOO and the main olfactory epithelium. To verify that the cells in sea lamprey were taking up the dye, sea lamprey were anesthetized (0.03% tricaine methane sulfonate (MS-222) for larva, 0.05% MS-222 for transformers, and 0.3% MS-222 for spawners, pH 7.4; Sigma-Aldrich, Oakville, ON, Canada). Once the lamprey became unresponsive by pinching the tail, the larval or other phases was removed from the MS-222 solution and put on a dry petri dish. The nasal pore was dried using a Kimwipe. The fixable dry crystal inserted using a needle or syringe; in some cases, a mixture of fixable dextran crystal plus 0.1% Triton-X plus 0.1 M NaCl (in deionized water) was also used. A 5-minute rest period followed to allow the dye to dissolve in the nasal cavity. During this time, chilled dechlorinated water was applied to the gills and tails. For the remaining phases of lamprey, a mixture of fixable dextran crystal plus 0.1% Triton-X plus 0.1 M NaCl (in deionized water) was deposited into the nostril using a needle and syringe followed by a 5-minute resting period. After the rest period, the specimen was returned to the holding aquaria for a minimum of 48 hours or up to a week. In attempts to improve techniques, dye was loaded twice with either a three hour wait, or full day wait in between the two dye loadings. The specimens were also put in chilled dechlorinated water odorized with 3kPZS & TCA or spermine & spermidine for 5 minutes before being placed in the holding aquaria. After a minimum of 48 hours, the lamprey was dissected and sectioned for further analyses.

3.2.6 Electroporation loading of dye into the main olfactory epithelium and AOO

The electroporation method has previously been used by Lukas (Weiss et al., 2019) on Xenopus nervous system successfully. Thus, individual sea lamprey of desired developmental stages was anesthetized using MS-222 dissolved in dechlorinated water at 6°C temperature. The nostril of the lamprey was brought into the field of view under a dissecting microscope and dried within the nostril and the surrounding tissue using Kimwipes to prevent the fluorescent dye from spreading. Using a thin needle or syringe either dry fixable dextran crystal, crystals mixed with Triton-X, crystals mixed with Triton-X odorized with 3kPZS or spermine was gently inserted into the nasal cavity. Two platinum wire electrodes with a diameter of 0.2 mm were carefully inserted with one electrode placed into the dye-filled nasal cavity ensuring no damage to the olfactory mucosa while the second electrode was placed either exterior or interior near the nostril. 20 to 25 Ampere current was applied either for 1 minute and 0.5 seconds of on-off pulses or continuous current (direct current). After removal of the electrodes, the lamprey was transferred into a beaker with chilled dechlorinated water odorized with the spermine and spermidine odorant and carefully placed in the holding aquaria for a minimum of 48 hours. The dextran dye entered the lumen of the main olfactory epithelium, then the olfactory sensory neurons cells where dextran was then transported along the olfactory nerve fibres, to the axonal terminals located in the olfactory bulb. After dissection, the spatial distribution of in the main olfactory epithelium, duct and AOO of the olfactory blub were observed under a fluorescence microscope (Figure 3.1).

3.2.7 Microscopy

Fluorescent sections were photographed using a Nikon Eclipse 800 epifluorescence microscope equipped with 10X to 60X oil immersion lens and a CCD camera (photometric

CoolSNAP HQ; Roper Scientific, Tucson, AZ). Image J was used to modify the levels brightness and contrast along with the cropping and merging of images.

3.3 Results

3.3.1 Identification of neuronal cells in the AOO

The AOO is located beneath the olfactory epithelium in caudoventral to the peripheral olfactory organ in all life stages of the sea lamprey (Figure 3.2a, Figure 3.3, Figure 3.4a, Figure 3.6, Figure 3.7a & b, Figure 3.8, Figure 3.11). The structure of the peripheral olfactory organ enlarges as the sea lamprey develops (Figure 3.3, Figure 3.4a, Figure 3.6, Figure 3.7a & b, Figure 3.11); in larva, the AOO consists of two small half-ring structures (Figure 3.3, Figure 3.9 b, c & d) whereas, in the transformer, parasite and spawner stage, the AOO has evolved into an enlarged ring with the ring size become larger as they approach later life stages (Figure 3.4a, Figure 3.6, Figure 3.7a & b, Figure 3.11, Figure 3.12c & d). The cells in the main olfactory epithelium of transformers were elongated and narrow (Figure 3.4b) in comparison to the cells in the AOO which were small and round-shaped (Figure 3.4c). Green, (2012) has previously mentioned that the cells were a rounder shaped in the AOO of adult sea lamprey, confirming the structure identified in the transformer. Immunolabeling with anti-acetylated tubulin in the peripheral olfactory organ of larval and transformer sea lamprey indicated that the cells in both the main epithelium and the AOO have a neuronal identity (Figure 3.3, Figure 3.4c) due to a single axon extending from the basal region of a sensory neuron, hair-like apical cilia on the cells with the nerve fascicles surrounding the main olfactory epithelium and the AOO were positively labelled with acetylated tubulin (Figure 3.3, Figure 3.4a, b & c, Figure 3.6).

3.3.2 GSIβ4 labelling of the duct in peripheral organ

GSIβ4 was used to map out the glomerular regions of the olfactory blub in larval sea lamprey and is also considered a biochemical marker for OSNs axon in lamprey (Frontini et al., 2003; Tobet et al., 1996; Zielinski et al., 2000). In the larval and transformer phase of sea lamprey, the lectin GSIβ4 was found at various locations including, the lumen side of the main olfactory epithelium, in the lumen of the AOO, and on the axons of the AOO (Figure 3.5d). Not all areas in the peripheral organ showed intense labelling of the lectin; there was not an intense presence of glycol conjugates in the main olfactory epithelium of transformer and spawner phase sea lamprey (Figure 3.5a, b & c, Figure 3.7b), however, the cells and the lumen side of the AOO strongly labelled with the lectin GSIβ4 (Figure 3.5e, 3.7b). The pathway sides connecting the lumen of the OE & the lumen of the AOO along with the entrance of the duct (open or closed, Figure 3.5a, b, c & d), Further, it has been observed that the duct elongates as the sea lamprey develops (Figure 3.5a to e, Figure 3.6, Figure 3.9b & c); in larva the duct was barely visible (Figure 3.9 b & c), but in the transformer the duct appeared small and round, or sometimes collapsed (Figure 3.5a to e). In parasites and spawner sea lamprey the duct had a corkscrew-shape, that was long and narrow (Figure 3.11a & b, Figure 3.6). The pathway in both the transformer and spawner phase lamprey contained carbohydrate-containing material (Figure 3.7a & b).

3.3.3 Cells within the AOO loaded with fixable dextran

Fluorescent dextran was used to load cells in the peripheral organ of larval, transformer, parasitic and spawner phase sea lamprey. It was observed that the OSNs in the main olfactory epithelium of all lamprey life stages were loaded with fluorescent dextran using the *in vivo* and electroporation method (Figure 3.9a, Figure 3.10a & b, Figure 3.11a, Figure 3.12a & b, not all

data shown). Furthermore, the presence of dextran in the glomerular regions of the olfactory bulb verify that the sensory neurons of the main olfactory epithelium were indeed taking up the dye as it was travelling through axons to the regions of projection in the olfactory bulb (Figure 3.8). Individual olfactory sensory neurons of the main olfactory epithelium were characterized in larval and transformer sea lamprey (refer to chapter 2 of the study, Grande, 2017).

Initially, the fixable dextran was delivered to cells in the AOO of sea lamprey by *in vivo* methods using either syringe or needle; however, the cells of the AOO did not load in all life cycle stages of the lamprey using this method (Table 1, Figure 3.10a). Electroporation was an alternative technique used to load cells in the AOO by applying fixable fluorescent dextran to the sensory neurons of the AOO using pulse or continuous currents (Table 2). During the larval stage, the dye was observed entering the lumen of the AOO occasionally but tended to stop at the entrance of the duct (Table 1, Figure 3.9b & c). Trials showed that few cells in the AOO were taking up the dextran using with pulsed and direct current (Table 1, Figure 3.9d). All trials performed transformer lamprey showed that the cells in the AOO failed to take up the dye with or without odorant current application using electroporation method (Figure 3.10). One trial performed on parasitic and spawner phase sea lamprey showed that the dye had entered the lumen of the AOO for the parasite and the cells within the AOO of the spawner lamprey (Figure 3.11c & d, Figure 3.12c & d, Table 1). The dye entered the lumen of the AOO via a duct in the parasite (Figure 3.9b, c, & d), but not all of the duct and lumen of the AOO showed the presence of dextran in the parasite (Figure 3.11a & d). Overall, the OSNs in the main olfactory epithelium were taking up dextran using both *in vivo* and electroporation; however, the cells of the AOO did not have successful loading in all life phases using *in vivo* techniques with or without odorants (Table 1). The electroporation application has shown some penetrability for the dye to enter the

lumen of AOOs and occasionally in the cells of the AOO during the larval stage (n= 7), spawned (n=1) and parasite (n=1) (Table 1).

3.4 Discussion

As the sea lamprey progresses through its developmental phases, the structure of the AOO also progresses through changes (VanDenBossche et al., 1997). The areas associated with the AOO and the AOO itself, enlarge and takes the shape of a ring structure connecting to the main olfactory epithelium with an elongated corkscrew duct. Although the size of the AOO is smaller in larva, this study indicates that OSNs within the AOO are still present and have a neuronal identity, which is also seen in other phases of sea lamprey. Previously, Green et al. (2012) indicated that the OSNs in the AOO of spawner send signals to the olfactory blub and have a complete set of functional units of sensory cells, including cilia and axons. It was also suggested that the duct also lengthened as the sea lamprey transformed from the larval phase to the spawning stage. Thus, larval and transformer phase lampreys would be the best model to observe the sensory responses within the AOO because the cells have complete functional component and the pathway is shorter to deliver dye into the cells of the AOO.

It has been investigated in this study that the duct of the transformer and spawner phase sea lamprey contains a lot of carbohydrate-containing material. The localization of GSIβ4 may indicates that environment of the duct and the AOO is different from the main olfactory epithelium. According to Pelosi, (1996), GSIβ4 labelling may indicate a specific environment for odoriferous molecules to dissolve in the peripheral olfactory organ of vertebrates and insects. It may indicate specific odorant can enter through the mucus layer to access the cells. There should be a way for odorants to enter the lumen of the AOO through the duct triggering the locomotion signal in sea lamprey.

To characterize the sensory neurons found within the AOO, different methods were applied in attempts to load fluorescent dextran into the cells of the AOO. It was observed that the OSNs of the main olfactory epithelium in larva and transformer consistently took up the dye as previously shown in the Grande, (2017) study. However, the question of how stimuli have access to the AOO remains puzzling. The fixable dextran did not enter the lumen or cells of the AOO in all life stages of sea lamprey using the *in vivo* method; the dye stopped at the entrance of the duct. Duct is preventing the entry of dextran into the lumen of the AOO. The duct may function as a regulator to deliver odorants into the lumen of the AOO. In the digestive system, the entering of food in the mouth triggers hormone release, which then controls the opening and closing of the duct during the digestion of food (Hsu et al., 2019). It has been previously stated that the AOO is surrounded by blood vessels and nonmyelinated nerve bundles (Hagelin & Johnels, 1955; VanDenBossche et al., 1997), and the presence of an odorant might trigger a hormone or neurotransmitter signal to open and close the duct during the sensory response. In this thesis, odorants were used in an effort to trigger the opening and closing of the duct, but dextran did not successfully enter the cells of the AOO and was impeded by the entrance of the duct.

Electroporation has been used to deliver a diverse range of molecules and materials into the intracellular space such as delivering genes to target cells, or fluorophores to living cells for real-time imaging without tissue fixation (Mehrle et al., 1985). The electroporation has fast delivery of molecules and increases the permeability of the plasma membrane (Pucihar et al., 2007). A previous study has shown the successful use of this method to study traces of the projections of receptors from the nose to the brain in Xenopus. The study also reported that they were successful in recording presynaptic input induced by stimuli on the dendrites of the

postsynaptic cells via functional calcium imaging (Weiss et al., 2018). Another study loaded calcium dye into the neurons of the central nervous system using electroporation and studied antidromic, reflex, and locomotor-like behaviours (Bonnot et al., 2005). In this study, electroporation was not successful in delivering dye into the cells in the AOO of sea lamprey; however, some specimen did take up fluorescent dextran with this method, but it was not consistent among many experiments.

In conclusion, the labelling of cells with acetylated tubulin and lectin indicated that cells of the AOO have neuronal identities; however, the pathway was too narrow and clogged with glycoconjugates molecule, preventing dextran from entering the lumen and cells of the AOO. Both techniques used in this study were not consistent with loading OSNs in the AOO. Different conditions for electroporation to deliver dextran molecules into the AOO were attempted, such as pulse parameters. In other studies, it has been indicated that the fraction of cell permeabilization and fluorescence decreased as cell density increased (Pucihar et al., 2007). Based on this information, perhaps carbohydrate-containing molecule within the duct may be shielding the AOO from the electric field, resulting in no dye take-up in the cell sort the pulsed amplitude was too weak that it prevented the dye from entering into the cells of larva, transformers, and parasites. The dilution of the dye and the swelling of cells caused after electro-permeabilization in tissues may also be attributed to low dye intake (Pucihar et al., 2007). There is a better need for new strategy to load cell in AOO.

Auxiliary mechanism for transporting external odorants to the receptors within the lumen of the AOO may also have some involvement in the difficulties faced. The AOO is similar to the vomeronasal organ, according to literature by Green, (2012) it may be possible to deliver dextran to the cells of AOO via the activation of nerves surrounding the organ or the dilation of the blood

vessel. A study done on the vomeronasal organ showed the in-flow and out-flow of fluids through the vomeronasal duct produced by nasopalatine nerve stimulation or atropine injection by dilating the surrounding blood vessels, the stimulation of these nerves might constrict the tissue surrounding the duct, resulting in the inflow of stimulus substances within the vomeronasal organ (Meredith et al., 1980; Meredith, 1994; Eccles, 1982) and may have the same effect on lamprey.

It is important to study how the sensory neurons in the AOO are detecting odorants. Studying this sensory organ may shed light on a tight connection with a general vs specific olfactory cue and a movement response. Other species that have more than one olfactory subsystem, usually have different cells detecting similar or different odorants, then sending signals to higher brain structures (Greer et al., 2016; Munger et al., 2009; Barrios et al., 2014; Liberles et al., 2014; Taniguchi et al., 2011). Fish that have a single olfactory epithelium have different cell types (ciliated and microvillous) detecting different odorants too (Sato et al., 2001). Similarly, the olfactory subsystem in sea lamprey also sends signals to different brain structures, but both main olfactory epithelium and AOO have ciliated cells. The AOO is very small in size in comparison to the main olfactory epithelium, and it only sends a signal to one medial glomerular region of the olfactory bulb which is responsible for detecting different types of odorants such as pheromones (3kPZS, PAMS-24 and PZS), and TCA & amino acids (Green et al., 2017; VanDenBossche et al., 1997). Thus, the AOO may only be responsible for detecting odorants that produce a motor response. The study of this subsystem in sea lamprey may provide insight on the evolutionary benefit of an AOO subsystem in sea lamprey and other species as the benefit of more than one olfactory system is not known.

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3.6 Figures

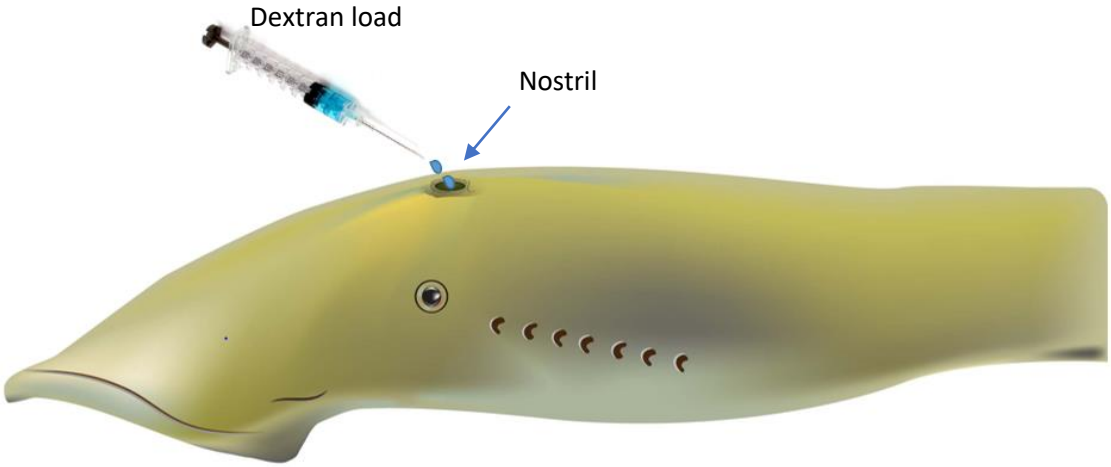

Figure 3.1: Florescent dextran loading into the nasal

Fluorescent dextran 488 or 568, 3000 MW was delicately loaded into the nostril using a long needle or syringe after the area was dried with a Kimwipe to avoid unwanted transfer of the dextran molecules in all life stages of the sea lamprey

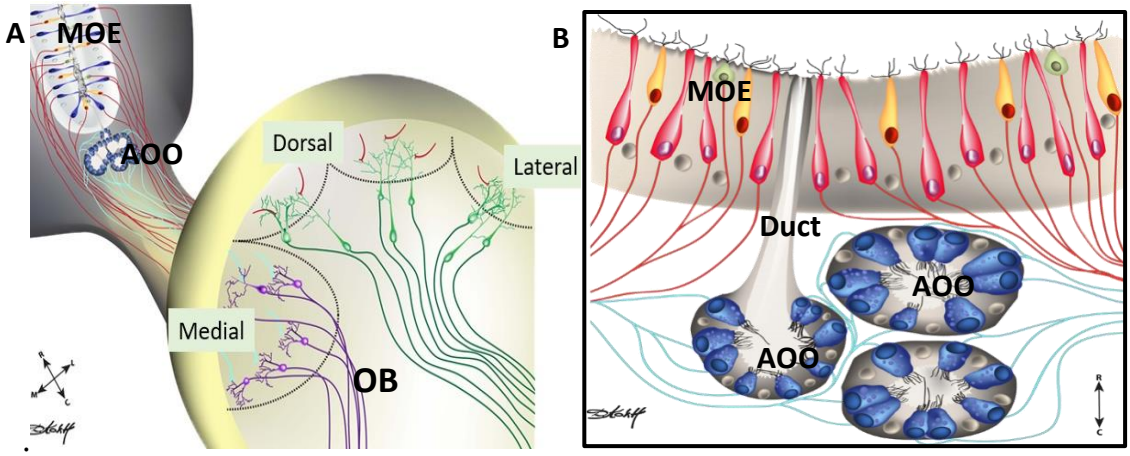

Figure 3.2: The main olfactory epithelium (MOE), accessory olfactory organ (AOO) and olfactory bulb OB) in sea lamprey

A) The peripheral olfactory organ contains the MOE and a narrow duct leading to the AOO. Nerve fibres can be seen (red) projecting from the MOE and AOO (light blue) into the olfactory bulb. (B) The AOO duct passes through the MOE into the AOO

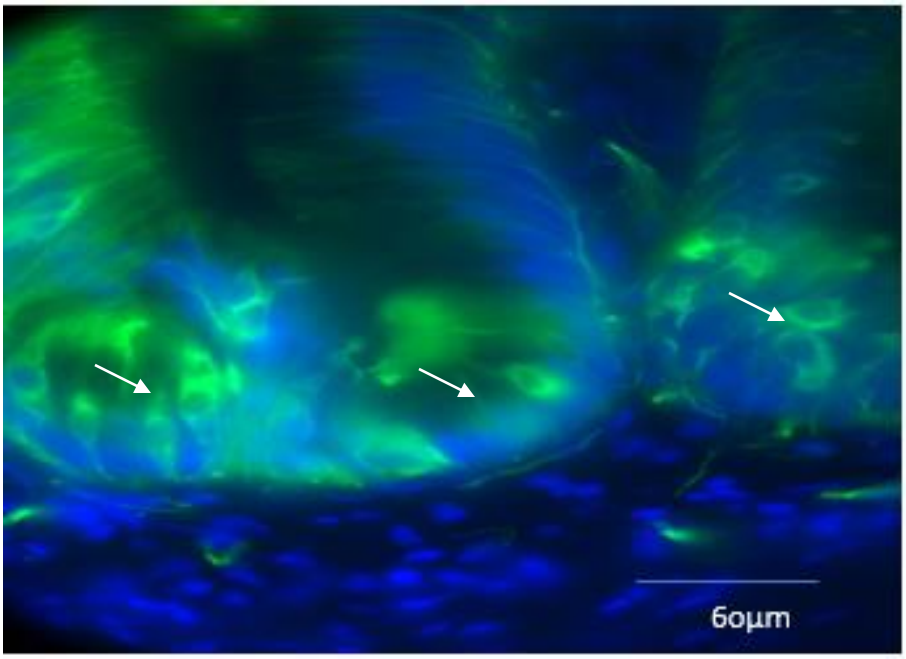

Figure 3.3: AOO cells labelled with acetylated tubulin in larva lamprey

The cells (arrow) of the AOO were successfully labelled with anti-acetylated tubulin (green). Describe their morphology. Blue is DAPI. Scale bar equal to 60 µm.

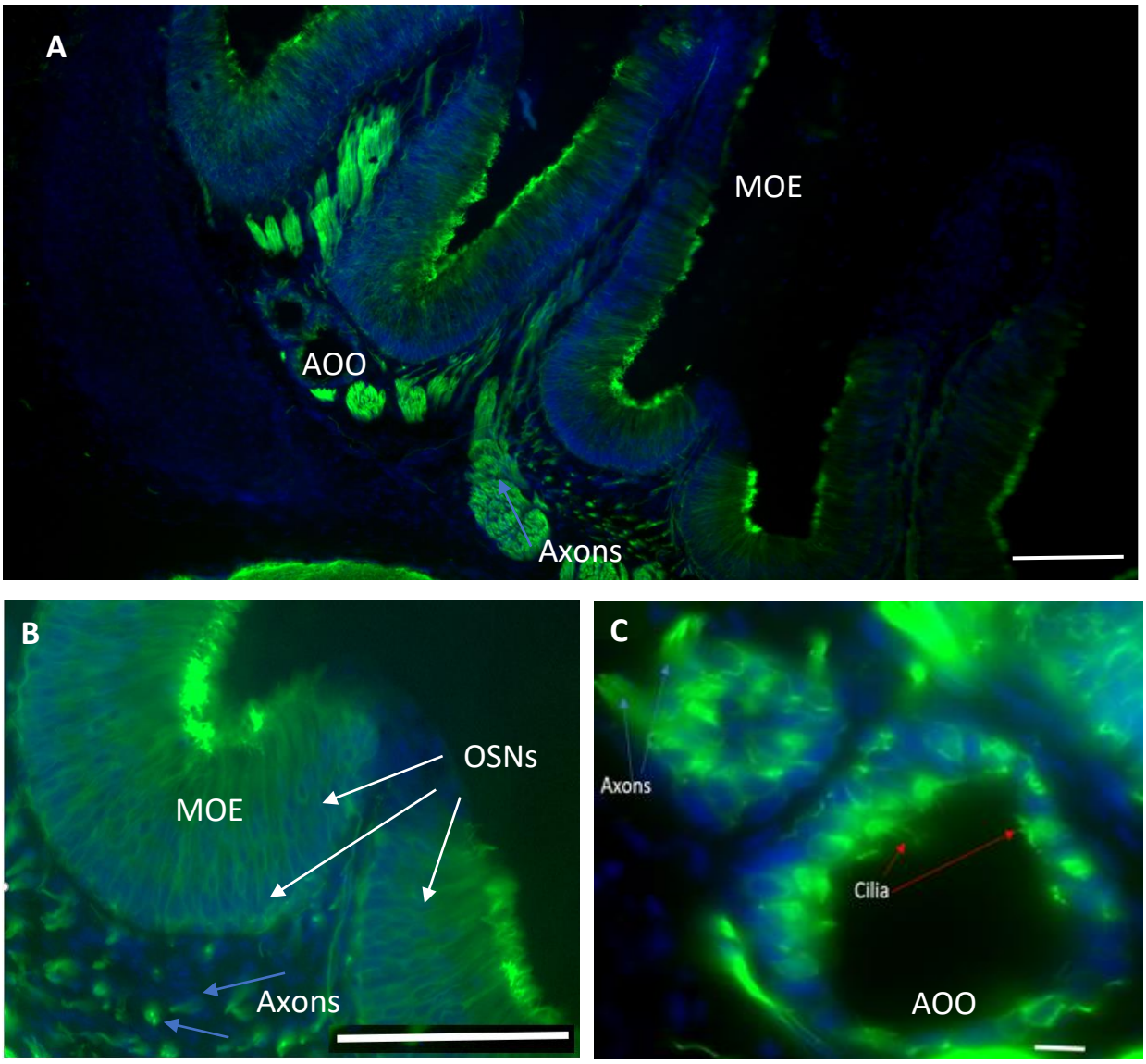

Figure 3.4: The cells within the main olfactory epithelium and AOO in the transformer sea lamprey

A) Cilia and axon bundles were labelled with acetylated tubulin (green). B) Olfactory sensory neurons (OSNs) labelled faintly with anti-acetylated tubulin in the main olfactory epithelium (MOE) and the AOO. C) The cilia and axons coming from cells in the AOO were immunolabeled with anti-acetylated tubulin. Blue indicates nucleus labelling with DAPI, the scale bar represents 60µm in both (A) and (B) and 20µm is C.

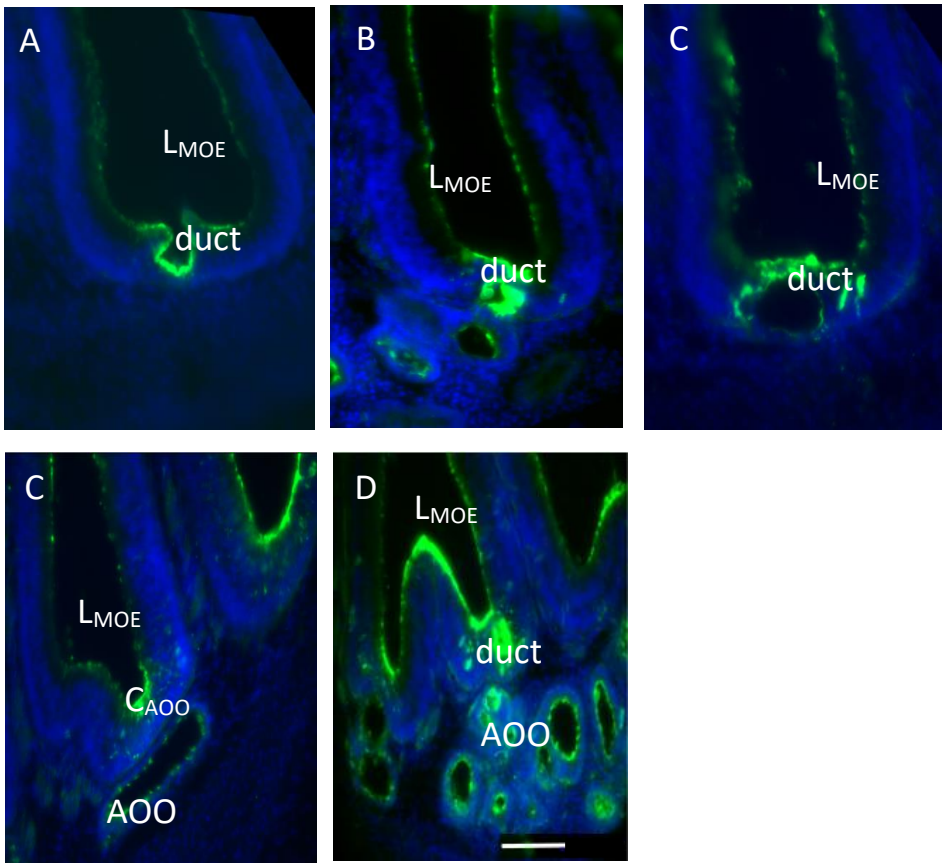

Figure 3.5: Labeling transformer main olfactory epithelium (MOE) and AOO with GSIβ4 lectin

Figures A through E indicate that the lumen of the peripheral olfactory organ (L_{MOE}), the duct and the lumen of AOO positively labeled with GSIB4 lectin. The lumen side of the AOO and the pathway connecting the lumen of the MOE and the AOO labelled with GS1B4 lectin (C_{AOO}). A) the duct appears small with the entrance opened, and side that connects the AOO is strongly reactive to lectin. B) the duct is small, collapsed and filled with glycoconjugates. C) The duct is small, with a closed entrance a strongly labelled with lectin. The duct appears to have a round D) the duct is not visible, the side connected to the epithelium to the AOO is labelled with GSIB4. The lumen and the cells in the AOO are also reactive to lectin. Blue indicates DAPI labelling (n=3 transformer, n= 3 larva). (Scale Bar A through E is equal to 60 µm

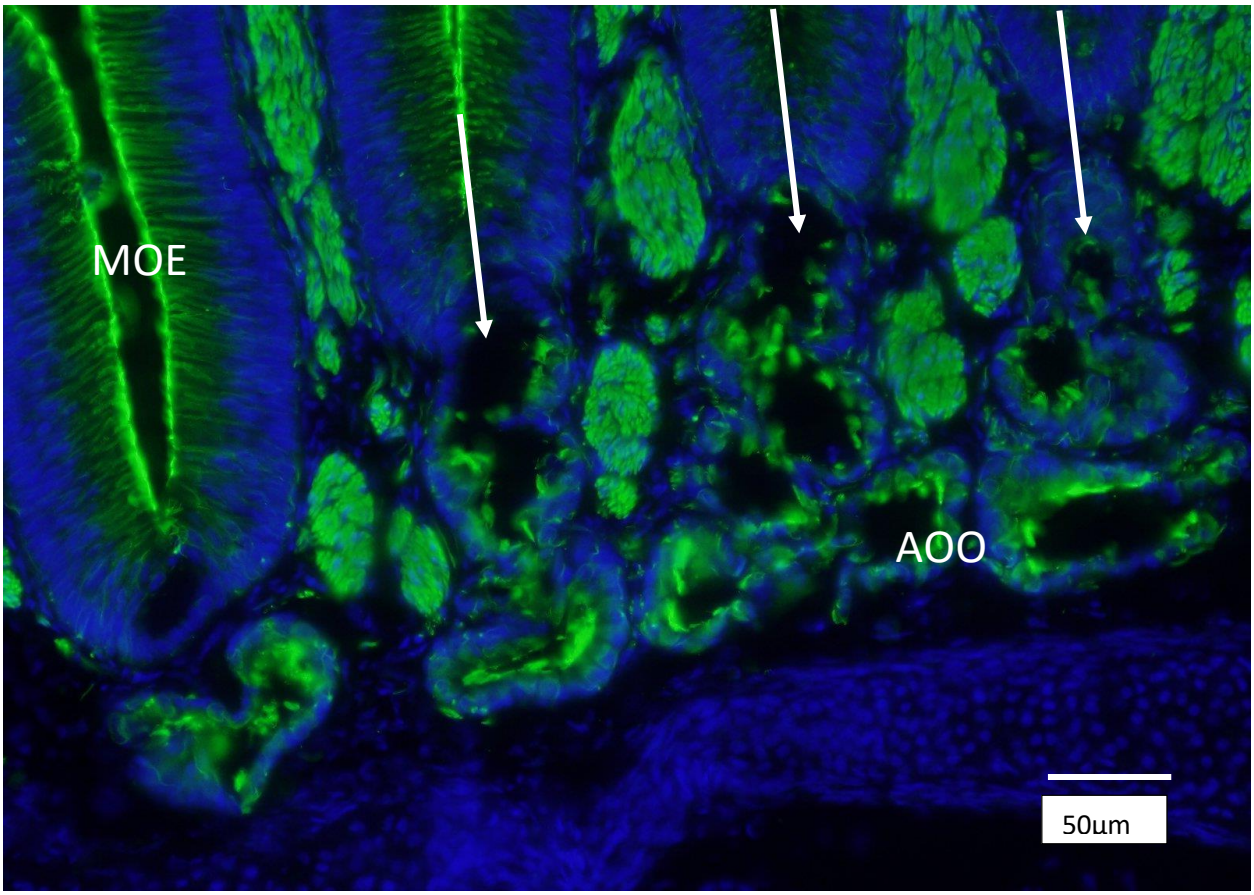

Figure 3.6: The duct in spawner sea lamrpey

The main olfactorey epithlium and the accessory olfactory organ were laeblled with anti-actylated tubulin (green). The arrow point to the duct in this figure. Blue is DAPI. Scale barrepresents 50 µm.

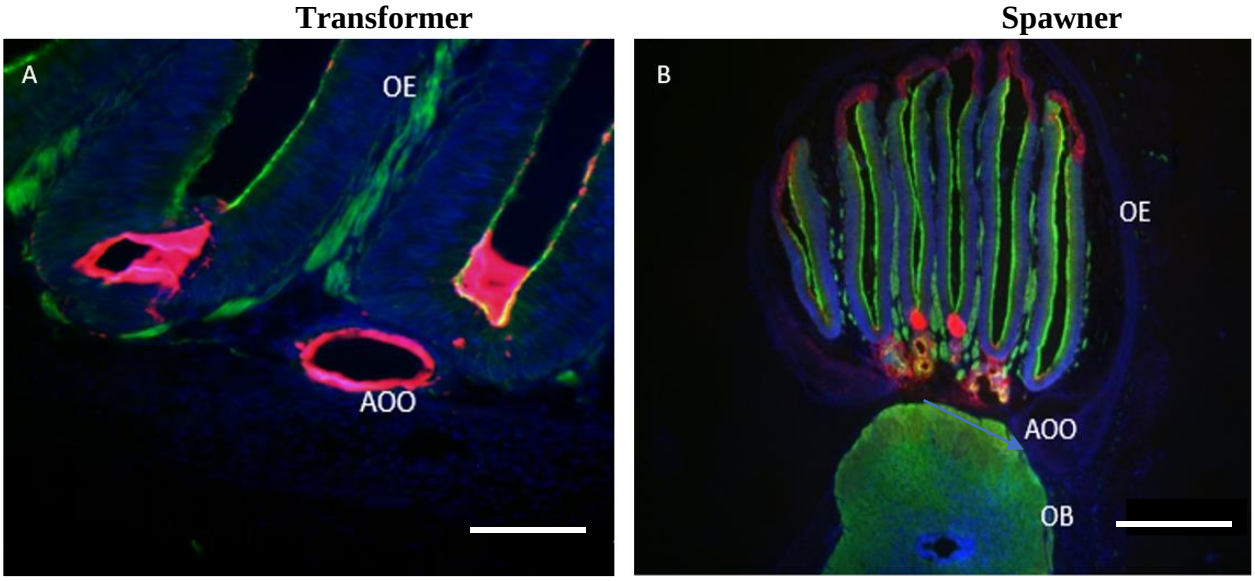

Figure 3.7: Labelling of lectin GSIβ$_4$ (red) materials adjacent to the AOO duct in transformer and spawning lampreys

GSIβ$_4$ labeled carbohydrate material located in the lumen between the AOO and MOE. (A: 60 µm B: 500 µm

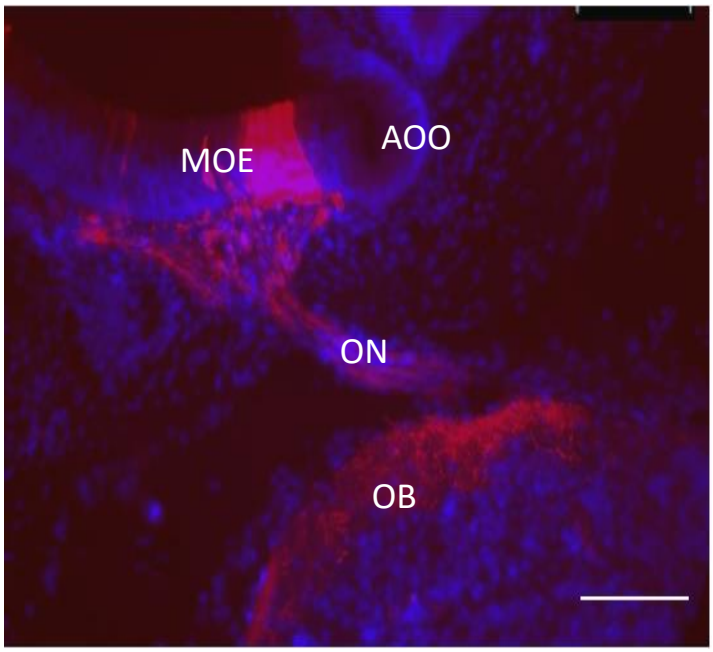

Figure 3.8: Larval peripheral organ, olfactory nerve, and the olfactory bulb loaded with fluorescent dextran

The olfactory sensory neurons of the main olfactory epithelium (MOE), the axons in the olfactory nerve (ON) and glomerular regions of the olfactory blub (OB), were loaded with dextran (red) that was applied through the nasal cavity. The cells in AOO were not loaded with fluorescent dextran. Scale bar is 60μm.

Larva

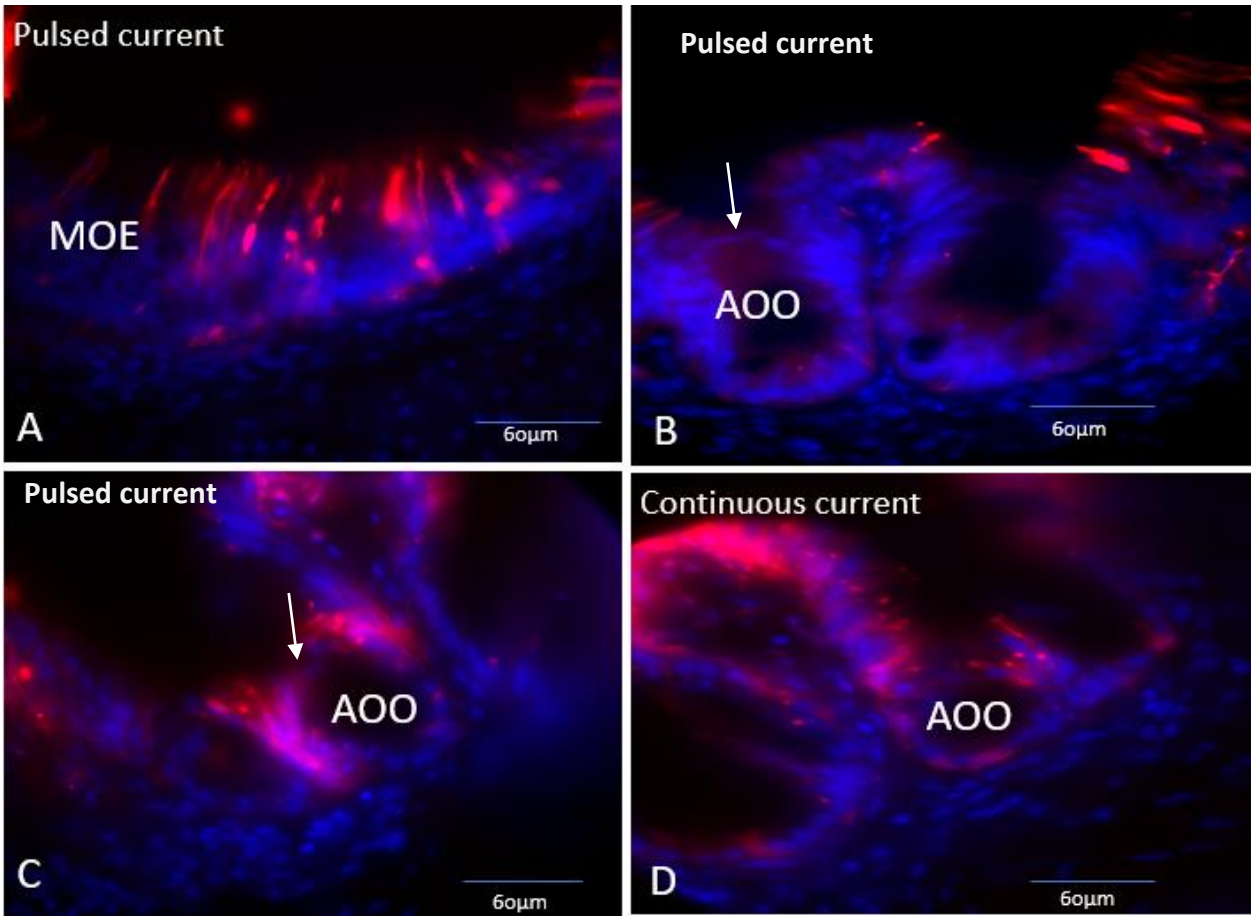

Figure 3.9: Anterograde loading of fluorescent dextran into the nasal cavity of larval sea lamprey following electroporation

A. The cells in the main olfactory epithelium (MOE) are loaded with fluorescent dextran (red), following pulsed-current electroporation. B. and C. Cells in the AOO are not loaded with fluorescent dextran (red) following pulsed-current electroporation. D. Cells in the AOO are loaded with fluorescent dextran upon application of continuous current. (Scale: 60µm).

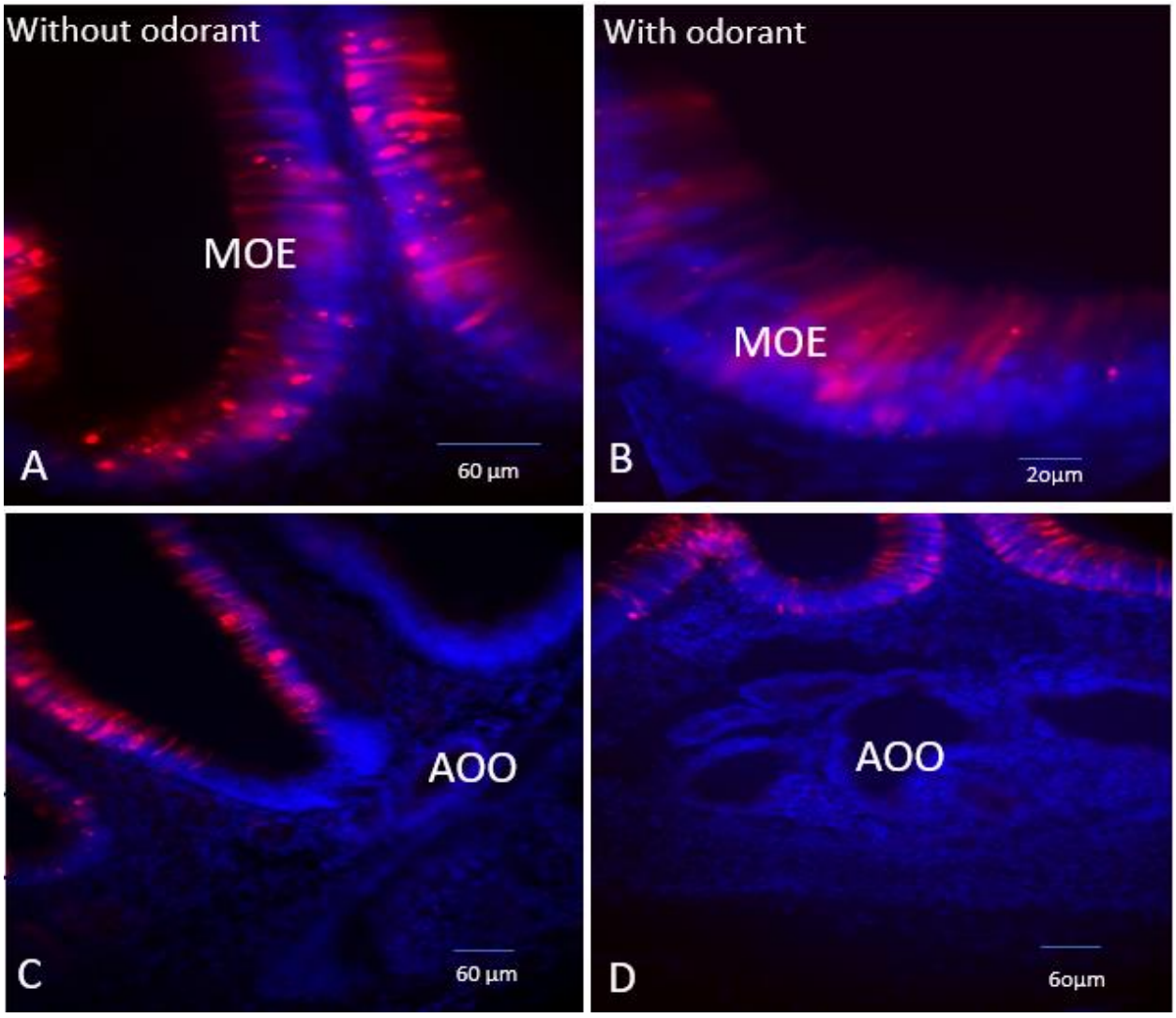

Figure 3.10: Anterograde loading of fluorescent dextran in the peripheral organ of ~~the~~ transformer lamprey following electroporation

In transformer sea lamprey, the main olfactory epithelium (MOE) was loaded. The AOO did not load following the injection of fluorescent dextran into the nostril, followed by electroporation with or without the odorant application**.** The fluorescent dextran is red, DAPI is blue (Scale bar: A, C and D is 60µm, and B is 20µm).

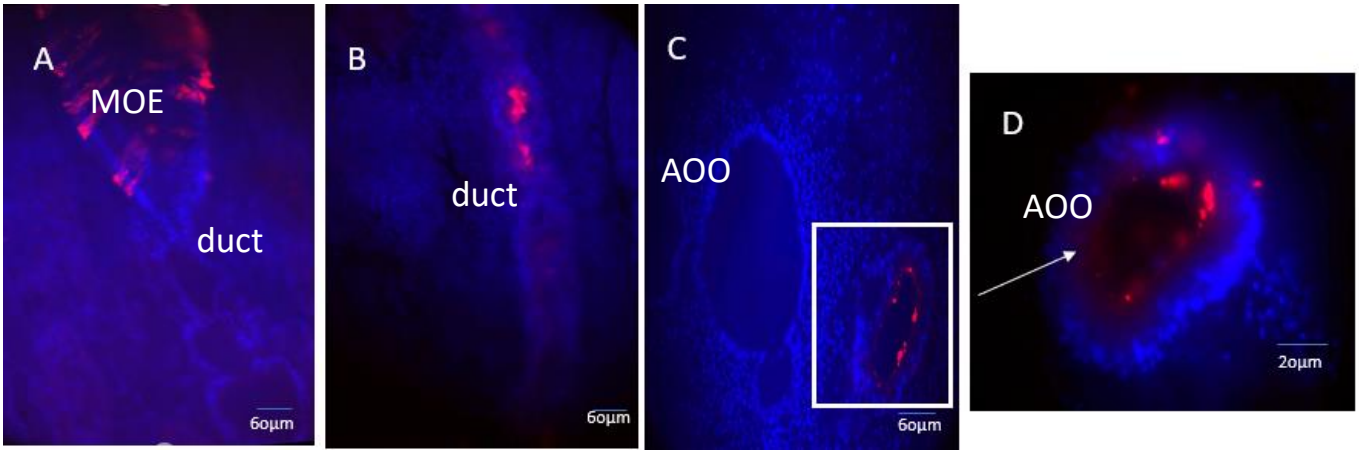

Figure 3.11: Anterograde loading of fluorescent dextran in peripheral organ of parasite following electroporation

In parasitic lamprey, the fluorescent dextran anterogradely loaded the cells in the main olfactory epithelium (MOE), the duct, and the lumen of the AOO, following two dextran applications and electroporation: A) Fluorescent dextran (red) labeled cells in the MOE, but not in the duct. B) The duct loaded with fluorescent dextran. C) & D) The dextran entered the lumen of the AOO but did not label the cells of the AOO. Scale Bar is A to C is 60 µm and D is 20 µm.

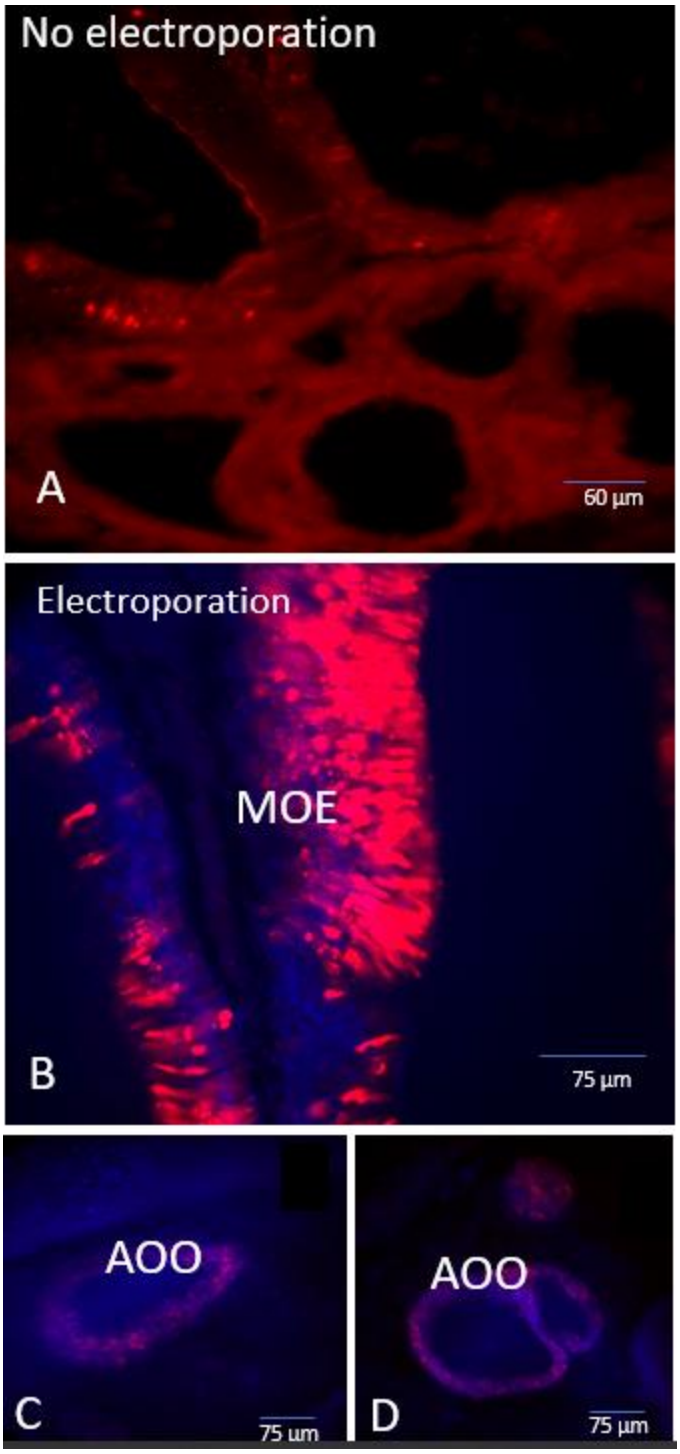

Figure 3.12: Red fluorescent dextran loading in the main olfactory epithelium and AOO of spawner sea lamprey

A) without electroporation labeling was sparse in the main olfactory epithelium and absent from the AOO. B) Following electroporation and a double application of dextran injection, the cells in the main olfactory epithelium were loaded with dextran. C) & D) show some fluorescence in the AOO following electrophoresis and a double application dextran injection with odorants. This labeling was successfully seen once out of three trials. Scale bar is A 60 µm and B, C, D are 75µm)

Stage	No Electroporation	Electroporation Pulsed current	Electroporation Continuous current
Larva (30)	-no load (8)	-no load (9) -AOO lumen loaded (5) -AOO cells loaded (1)	-no load (4) -AOO cells loaded (3)
Transformer (17)	-no load (8)	-no load (6)	-no load (3)
Parasite (3)		-no load (2) -AOO lumen loaded (1)	
Spawner (8)	no load (4)	-no load (3) -AOO cells loaded (1)	

Table 2: Fluorescent dextran loading of the AOO

The first column indicates the sample size (in brackets) and stage of the sea lamprey, the second column indicates fluorescent dextran was loaded into the nasal cavity by needle or syringe without electroporation. The dextran crystals were used with or without triton x-100 permeabilizing the plasma membrane of cell. Dextran was injected twice along with odorant application to aid with the take up of dextran. No cells within AOO loaded with dextran. The third column indicates that the fluorescent dextran was injected, followed by pulse current electroporation to facilitate dextran take up. In 5 instances dye was seen in the lumen of the AOO in larval lamprey, 1 instance in parasites when the dye was injected twice, and 1 instance in spawners when the dye was injected twice. The fourth column indicates that the experiment was done using continuous current instead of pulsed current; there were three instances when the dye entered the cells of the AOO in larval lamprey.

Chapter 4: Neuronal fibers within the dermal papillae of different life stages of the sea lamprey

4.1 Introduction

Chemoreception is not restricted to the olfactory system; the taste system and diffuse chemosensory systems are also involved in detecting various external stimuli to produce behavioural and physiological responses. The sea lamprey utilizes the diffuse chemosensory system to detect multiple chemical stimuli in their surrounding environment such as amino and sialic acids, body mucus (Daghfous et al., 2019; Baatrup & Doving, 1985). Whitear & Lane, (1983) were the first to observe the sensory cells on dermal papillae in the diffuse chemosensory system of Brook lamprey and anadromous River lampreys. They used transmission electron microscopy to observe and describe "oligovillous cells" on protruding dermal papillae that spread along the caudal border of the adult and mature river lamprey gills (Whitear et al., 1983). Whitear et al., (1992) also reported that the nerve fibers approach at the base of SCCs in a spur-like process with a number of tapering divisions. The oligovillous cells are also referred to as solitary chemosensory cells (SCCs) because they are found as isolated cell with microvilli in the epithelia of many aquatic and territorial species. SCCs have been reported in the oral and nasal cavity of cows (Tizano, 2006), the nasal epithelia of humans (Hansen et al., 2005), the nasal epithelial (especially the VNO), and vallate oral papilla of rodents (Finger et al., 2003; Sbarbati & Osculati, 2002, 2004). SCCs were also found in pectoral fin of the sea robin (Whitear, 1971; Silver & Finger, 1984), and the anterior fin of rockling (Whitear et al., 1988, Bardach & Case, 1965).

SCCs found in different species have the capability to send a signal to the central nervous system. According to Kinnamon, (2012), the epithelium where the SCCs are found is densely packed with nerve fibres and nerve endings which relay signals to higher brain structures.

In the mammalian respiratory system, SCCs are innervated by the trigeminal nerve which monitors potential toxic compounds in the surrounding environment (Finger et al., 2003). In the nasal cavity of rodents where airflow in and out of the nose brings many different chemicals, the SCCs detect high levels of irritants and bitter compounds (Finger et al., 2003, Ougra et al., 2010). In rodents, the SCCs release the neurotransmitter acetylcholine to nerve fibres and produce nociceptive reflexes such as the sneezing response (Saunders et al., 2014; Finger et al., 2003; Sliverman, 1989, Ougra et al., 2010). In rockling (a teleost fish), the dorsal fins are innervated with recurrent facial nerves where nerve fibers were placed as nerve bundles among the bases of sensory cells (Whitear et al., 1988). SCCs on the surface of these fins in rockling are known to sample water (Kotrschal et al., 1993, 1998; Kotrschal & Whitear, 1988; Peters & Kotrschal, 1987) while the rockling swims, the SCCs can detect incoming predators or prey by sensing their body mucus, containing alarm responses (Chia et al., 2019) and bile acids (Kotrschal, 1995, 1996). In another teleost fish, the sea robin, SCCs are also found on fins and activated by food-related stimuli such as amino acids and other molecules from marine invertebrates. The spinal nerves were located near the SCCs on the fins of sea robin and maybe involved in controlling locomotor mediated behaviour in sea robin (Whitear, 1992; Silver & Finger, 1984; Kotrschal, 1995, 1996). The SCCs are different with respect to species, location, and type of nerve fibers that the SCCs are innervated with (Finger, 1997). Due to this, SCCs are highly specialized cells that may induce different behavioural responses depending on the type of nerve fibres the SCCs are innervated with, and the regions the SCCs are associated with.

Given that each phase of the sea lamprey life cycle: larva, transformer, parasite and spawner, serve a different role, it is vital to find the location of SCCs in each phase. The role of the SCCs in sea lampreys is not yet known. It is known that the SCCs are present in the oral disc,

nostril, the gills, and fins of the adult sea lamprey (Daghfous et al., 2015, 2019, Suntres et al., 2016). Studies by Daghfous et al., (2019) and Muhammad et al., (still in progress) showed neural responses from the dermal papillae of spawner sea lamprey responding to dead trout water, amino acids, taurocholic acid and its derivatives. The SCCs may be playing a role in water sampling, have a food-related function, or be utilized for predator avoidance. Suntres et al., (2019) found that SCCs are located on the dermal papillae of gills in parasitic and mature sea lamprey and that the nerve fibre are found near the base of SCCs in the gills of spawner sea lamprey. The presence of these fibers near SCCs on the gill dermal papillae suggests that the SCCs transmit signals via nerve fibres to the central nervous system. Suntres et al., (2016) and Daghfous et al., (2019) also found the presence of SCCs on the dermal papillae of the oral disc, nostril, fins of adult sea lamprey and the gills of transformer sea lamprey. She also indicated that the fins of transformers have indents but no SCCs, whereas the oral disc of transformers has protruding tissues. Suntres, (2019) reported that the outer ring of the oral disc on transformers has protruding tissues, which may be fimbriae or papillae (Dawson, 1905; Cook et al., 1990; Khidir & Renaud, 2003); according to Cook et al., (1990) and Khidir & Renaud, (2003), fimbriae are shaped as irregular leaf-like structure, involved in helping the fish to create a secure suction on its prey and the papillae are shaped as finger-like structures to collect sensory information. In spawners, both fimbriae and papillae could be easily distinguished by its shape under a microscope; however, in transformers, the fimbriae or papillae could not be distinguished because of the smaller structure of protruding tissue. Suntres et al., (2019) did not study if SCCs were present in the oral disc of larva, or the oral disc and nostril of transformers and parasites, but an experiment conducted by Whitear & Lane, 1983 indicated that SCCs were present in larval *Lampetra Planeri*, underneath the oral hood. No study has been published about the

neuroanatomy of the dermal papillae on the oral disc, nostril or fins of transformers and parasites to date.

Daghfous et al., (2019) reported that the nose and oral papillae are innervated by the trigeminal nerve, the gill papillae are innervated by the glossopharyngeal or vagus nerves and the dorsal fin papillae are innervated by the spinal dorsal roots (Daghfous et al., 2019). These nerve fibers may have an association with Merkle cells or other types of receptors cells according to Daghfous et al., (2019). Daghfous et al., (2019) do find Merkle cell in oral disc, nostril, fins and gill of spawner phase sea lamprey. To find that the nerve fibers, found within the papillae are innervated with SCCs, we need to used technique that can identify nerve fibers approaching in all regions of sea lamprey. Acetylated tubulin, an antibody, has previously been used to label the nerve fibers approaching the base of taste bud in sea lamprey (Barreiro-Iglesias et al., 2008, 2009). Suntres et al., (2019) indicated that the nerve fibers are found at the base of SCCs in the gill papillae by labeling with acetylated tubulin, but it has yet to be confirmed the nerve fibers are found within the nostril, oral disc or fins papillae associated with SCCs. Immunolabeling technique that label the nerve fibers with acetylated tubulin, can be applied towards these regions. By finding nerve fibers near SCCs, it could determine whether these cells have the capability to send signals via nerve fibers to the nervous system where signal processing occurs. The goal of this study is to gain an understanding of SCCs during the lamprey life cycle by systematic investigation of their occurrence on dermal papillae, and the innervating fibres during the different stages of the sea lamprey life cycle.

4.2 Methods

4.2.1 Animal collection

All procedure was done according to guidelines provided by the Animal Care Committee of the University of Windsor and the Canadian Council on Animal Care. Larva, transformer, parasites, and spawning sea lamprey were collected from Hammond Bay Biological Station, Millersburg, MI and post-metamorphic (transformer-phase) lamprey were supplied by ACME Lamprey Company, Harrison, ME. All animals were housed at the University of Windsor in aquaria at 6°C ± 1 °C in dechlorinated water under static renewal conditions.

4.2.2 Tissue preparation

Sea lampreys were anesthetized in a solution of tricaine methanesulphonate (MS222, 0.05 g in 100 ml or 0.5 g in 2 L), mixed in dechlorinated water and pH to 7.4 using 2 drops of 5 M NaOH, followed by decapitation before the first brachial pore. The gills, oral disk, nostril and dorsal fins were removed from the rest of the body and fixed in 4% PFA in a separate container for 6-18 hours. The tissues were then incubated in 20 % sucrose overnight and sectioned at 18-20 µm on Superfrost plus slides (Fisher Scientific) using Leica CN3050 S cryostat.

4.2.3 Staining of microvillous cells using phalloidin

The sectioned tissue was covered with a drop of phalloidin (5µm in 395µL of 0.1M PBS, Life Technologies, Alexa 488 A1372) for 30 minutes before being cover-slipped with VectaShield (H-1200/ H-1800, Vector Laboratories, Burlingame, CA) mounting medium with DAPI counterstain to label nuclei.

4.3.4 Immunohistochemistry

Acetylated tubulin has previously been used to study the structure of taste buds in brook and river lamprey. This technique is usually used as a ciliary marker (Baatrup, 1983a, & b, 1985b) or a general marker of neuronal-like elements in the nervous (Piperno & Fuller, 1985; Barreiro-Iglesias et al., 2008a, & b). For acylated tubulin labeling, the sections were immersed in acetone for 10 minutes at -20 °C, followed by rehydration for 10 to15 minutes in 0.1 M PBS. Cryosections were then incubated in acetylated tubulin (1/1000 in 0.1 M PBS plus 5% goat serum, sigma, pH 7.4) for two to three days at 4°C on shaker. Slides were then washed every 30 minutes 3 times while remaining on the shaker at 4°C before moving sections into Alexa 488 conjugated anti-mouse secondary antibody (1: 250 in 0.1 M PBS; A21151 Invitrogen) for one to two days. After this incubation period, the tissues were washed with 0.1 M PBS, coverslipped, and mounted with Vectasheild.

Calretinin has been used as a marker for amphibian taste buds, taste cells in teleost fish, and SCCs in Big-headed carp (Hansen et al., 2014; DíazRegueira et al., 2005; Northcutt, 2005). For calretinin labelling, the sections were immersed in acetone for 10 minutes at 20°C followed by rehydration for 10 to 15 mins in 0.1 M PBS. Sections underwent 25 minutes of antigen retrial (2.94 g of Trisodium citrate in 1 L deionized water plus 0.01 % Tween-20, pH to 6) at 90°C in a hot water. Cryosections were then moved into the primary antibody rabbit polyclonal anti-calretinin (1: 500 0.1 M PBS plus 5 % goat serum, Swant,7699/3) for three days at 4°C on a shaker. Slides were then washed every 30 minutes for 3 cycles while on the shaker at 4°C and then incubated in Alexa 488 conjugated anti-rabbit secondary antibody (1: 250 in 0.1 M PBS; A32731 Invitrogen) for two days. After incubation, sections were washed with 0.1 M PBS, coverslipped, and mounted with Vectasheild.

4.3.5 Microscopy

Fluorescent sections were photographed with a Nikon Eclipse 800 epifluorescence microscope equipped with 10X to 60X oil immersion lens and a CCD camera (photometric CoolSNAP HQ; Roper Scientific, Tucson, AZ). Image J was used to modify parameters such as brightness, contrast, image crops, and merges.

4.3 Results

4.3.1 Dermal Papillae during sea lamprey life cycle

Papillae were found underneath the oral disc hood of larva; however, with the gills, nostril and fins, no papillae were found (Table 3). In transformers, small bud-like papillae were found on the gills (previously mention by Suntres, 2016) and nostril (Table 3, Figure 4.1a). The protruding tissues found on the outer ring of the oral disc in transformer, may be fimbriae (Figure 4.1b, Table 3). Phalloidin labeling may indicate whether the protruding tissues found on the oral disc is papillae by checking the presence of SCCs on this tissue. The dermal papillae were absent from the fins of transformers (Table 3, figure 4.1c), but were found on the gills of parasitic lamprey (Figure 4.5, Table 3). In spawners, the dermal papillae were located on the outer boundaries of the oral disc, the caudal side of the gills, the outer periphery of the nostril, and the posterior edge of the caudal fins (Figure 4.2). For spawners, the dermal papillae on the oral disc can be distinguished from fimbriae under a stereoscope by its triangular node shape (previously mention by Suntres, 2016 too).

4.3.2 Localization of SCCs on dermal papillae in different regions

Phalloidin was used to observe SCCs regionality during the different life stages of lamprey. Phalloidin was used to locate SCCs on papillae by labelling actin which is present in microvilli and actin filaments found adjacent to the cell membrane and epithelial cells,

distinguishing the spindled-shape SCCs (Whitear & Lane, 1983; Suntres et al., 2019) from other epithelial cells. The microvilli-like cells, solitary chemosensory cells, are found on larval oral disc (n=8) (Figure 4.3a & b, Table 3), transformer gills (n=6), nostril (n=2) and the oral disc (n=4) (Figure 4.4a, b, c & d, Table 3), the gills of parasites (n=3) (Figure 4.5) and in spawner gills (n=12), oral disc (n=5), fin (n=5) and nostril (n=5) (Figure 4.6a, c, b & d, Table 2). In transformers, the labelling of SCCs with phalloidin on the protruding tissues of the oral disc indicate that dermal papillae were present on the oral disc of the transformer.

4.3.3 Epithelial nerve fibres within dermal papillae

The dermal papillae and SCCs were found on the oral disc of larva (n=6), and the oral disc, nostril, and gills of transformers (n=5). In parasite, it was found on the gills (n=4), and in spawner, it found on the oral disc (n=10), nostril (n=8), fins (n=8) and gills (n=14) (Table 3). Acetylated tubulin was used to observe the spatial relationship between nerve fibres and the SCCs. Nerve fibres appeared to be in lamina propria of papillae found in larval sea lamprey, these nerve fibres were seen to enter the epithelial region located near the base of SCCs in larva. (Figure 4.3a & b). In transformers, nerve fibres appeared to be in the lamina propria of gills, the oral disc and nostril (Figure 4.4a, b, c & d). Within the gills of the transformer, the nerve fibres were found at the basolateral region of the SCCs (Figure 4.4a & b), nerve fibres were also seen in the dermal papillae of parasites (data not shown). In spawners, the epithelium dermal papillae were innervated with a lot of nerve fibres that were seen at apex tip of each region (gill, oral disc, nostril and fins) of papillae (Figure 4.7a, b, c & d).

Suntres et al., (2019), had previously investigated the dermal papillae found on the gills of spawner labelled with the calretinin antibody. Calretinin has been used to label taste cells in sea lamprey (Barreiro-Inglesias et al., 2008), the retina of lamprey (Villar-cheda, 2006), and

SCCs of Big-headed carp (Hansen et al., 2014). The oral disc of larva, the gills of transformers, and the fins, nostril and oral disc of spawner did not label with calretinin (Table 3). The SCCs within gills of the parasites and spawner successfully labelled with the calcium binding protein calretinin (Figure 4.8, Table 3).

4.4 Discussion

This study indicated that papillae were present in the oral disc of larva, the oral disc, nostril, and gills of transformers, the gills of parasites, and the oral disc, gills, nostril and dorsal fins of migratory sea lamprey. Each dermal region showed the presence of solitary chemosensory cells in all sea lamprey life stages. In larva, the papillae were found underneath the oral disc hood, which mimics Lane & Whitear, (1982) who also found papillae with numerous SCCs present in larval of *Lampetra Planeri*, underneath the oral hood. It was expected that papillae would be found underneath the oral hood of the larva because water flows through the oral disc and out of the gills (Rovainen, 1975). According to Kotrschal, (1995), SCCs location in aquatic species seems to be towards flowing water over the surface of the body. In rodents, SCCs are found at sites where airflow in and out of the nostrils occur before VNO (Finger et al., 2003; Ogura et al., 2010). The SCCs on the larva oral disc may be involved in avoiding predators and avoiding harmful substances. There was a lack of papillae found on the fins, oral disc, and nostril of larva which may be attributed to staying buried in sand, thus not having a need for these structures to probe water at this life stage.

Since transformers enter open water, there should be a way to probe water to avoid predation or irritant substance to enter while trying to secure a food source. During this stage, they no longer filter-feed, and they have the ability to breathe through gills and use their oral disc to attach to prey (Randall, 1975). The presence of papillae and SCCs on the outer boundaries of

the oral disc and gills in transformers indicate that these structures face the flow of water and contact the surrounding environment first to induce appropriate behavioural responses (Cook et al.,1990; Daghfous et al., 2019). The presence of papillae on the oral disc and gills may be involved in detecting incoming prey and predators by detecting their body mucous as it has been shown in a study that fish body mucus has an alarm substance that elicits an alarm behaviour response in Zebrafish (Chia et al., 2019). No physiological research has been done on transformers oral disc and gills due to its small structure and the low number of SCCs, previously mentioned by Suntres, (2016). According to Baatrup & Doving, (1985b), the SCCs on the oral disc of adult brook lamprey respond to dead trout water and sialic acid, while Daghfous et al., (2019) found that the gills of adult sea lamprey elicit an excitatory response to glycine, proline and TCA, and an inhibitory response to arginine and serine, with no response to 3kPZS or sex pheromones. Previously, SCCs were found in the nose of the mammalian system and the nostril of adult sea lamprey (Daghfous et al., 2019; Finger et al., 2003). According to Finger et al., (2003), the SCCs, found in the nose of rodents served the purpose of detecting toxic substances or irritants initiating the sneezing reflexes (Finger et al., 2003; Ogura et al., 2010), as such the nostril of transformers may be involved in detecting toxic or irritating substances.

Both parasites and spawners have important behavioural roles; parasites search for food while navigating their surrounding environment avoiding danger, while spawner migrate downstream to find the breeding area and mates. Their diffuse chemosensory system may potentially be helping to collect more information, similar to the olfactory system that induce locomotor-mediated behaviour. During spawning, lamprey pick up rocks and construct nests in riffles using their oral disc. They may use the SCCs to find a clean area, devoid from toxic substances. Once a female has been attracted, the males attach their oral disc with the head of the

female lamprey while intertwining together during mating (Applegate, 1950; Johnson et al., 2015). The oral disc may have a role in probing water to find a suitable habitat or mate. The presences of SCCs on the dorsal fins of sea lamprey maximize spatial information from their surrounding environment while swimming. In other species, such as rockling that used undulating dorsal fins to detect upcoming fish (Kotrschal, 1995). SCCs on fins of rockling may bear similarities with the dorsal fin in lamprey for detecting prey and predator in the surrounding area.

This study reported that the dermal papillae were innervated with nerve fibres in the oral disc of the larva and the oral disc, gills and nostril of transformers, and the oral disc, gills, nostril and dorsal fins of spawner sea lamprey. It is essential to find the spatial location of the nerve fibres near SCCs because it will indicate whether SCCs can send sensory information to higher brain structures. In larva and transformers, the nerve fibres were found at the basolateral side (adjacent area) of SCCs, but in spawners, the epithelium of dermal papillae in any region was innervated with nerve fibres wherever SCCs were found. Kotrschal et al., (1993) had reported that the skin of Gadid fish Ciliata Mustela (Teleostei) was innervated with spinal and facial nerves, and spinal nerve fibres were also found innervating SCCs the fins of sea robins which plays a role in detecting upstream stimuli, whereas the trigeminal nerve fibres innervate SCCs found in rodent airways detecting bitter and harmful substances. SCCs play a different role in different species depending on the types of nerve fibres located at the base of the cell; Dagfous et al., (2019), injected tracers into different papillae discovering that the trigeminal nerve innervated the oral and nasal papillae, the glossopharyngeal and vagus nerves innervated the gill pore papillae, and the dorsal spinal roots innervated dorsal fin papillae. Since the different types of nerve fibres were found in the epithelium of dermal papillae where SCCs are located, perhaps

the oral disc, nostril, gill and dorsal fins may be involved in collecting information inducing different functions in sea lamprey. The successful reproduction and survival of sea lamprey may depend on the diffuse chemosensory system and the olfactory system collaborating to be able to thrive in the Great lakes.

This study found that the SCCs on the dermal papillae of gills in the parasite and spawner sea lamprey labelled with the calcium binding protein, calretinin. The calcium-binding protein is usually involved in regulating the intracellular calcium storage at synapses (Diaz-Regueira et al., 2000; Levanti et al., 2008), and the labelling of epidermal cells with calretinin occurs in SCCs, suggesting that SCCs are neural cells (Villar-Cheda, 2006). Positive calretinin labelling was reported in the taste system of lamprey and other vertebrates, suggesting homology to the taste system (Barreiro-Iglesias et al., 2008c, Hansen et al., 2014; Saunders et al., 2014). In other word, both labelled with calretinin, may share common ancestry or structure and function. Overall, this study suggested that the diffuse chemosensory system may be functional in all life stages, since SCCs were found to be innervated with different types of nerve fibres which may be sending information to a different part of the brain resulting in a variety of behaviour responses. For future, it would be essential to discover stimuli that induced a response in these cells. The discovery of stimuli that induces responses in these cells may contribute to providing population control methods for the Great Lakes.

4.5 References

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4.6 Figures

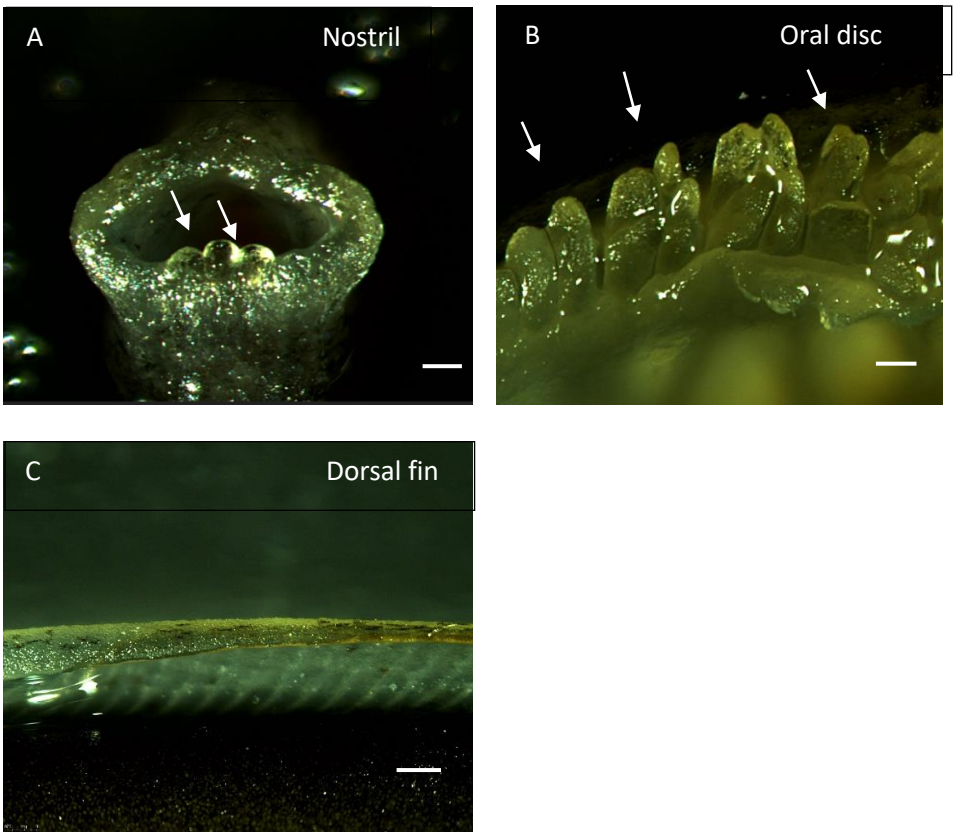

Figure 4.1: Stereomicrographs of the nostirl, oral disc and fin of a transformer

Protruding tissue denoted by arrows are located on the transformer nostril and oral disc, this tissue may be dermal papillae.The protruding tissue seen on the nostril (A) and oral disc (B) was not seen on the dorsal fin ©. Scale bar A) is 100 µm and B and C) 250 µm

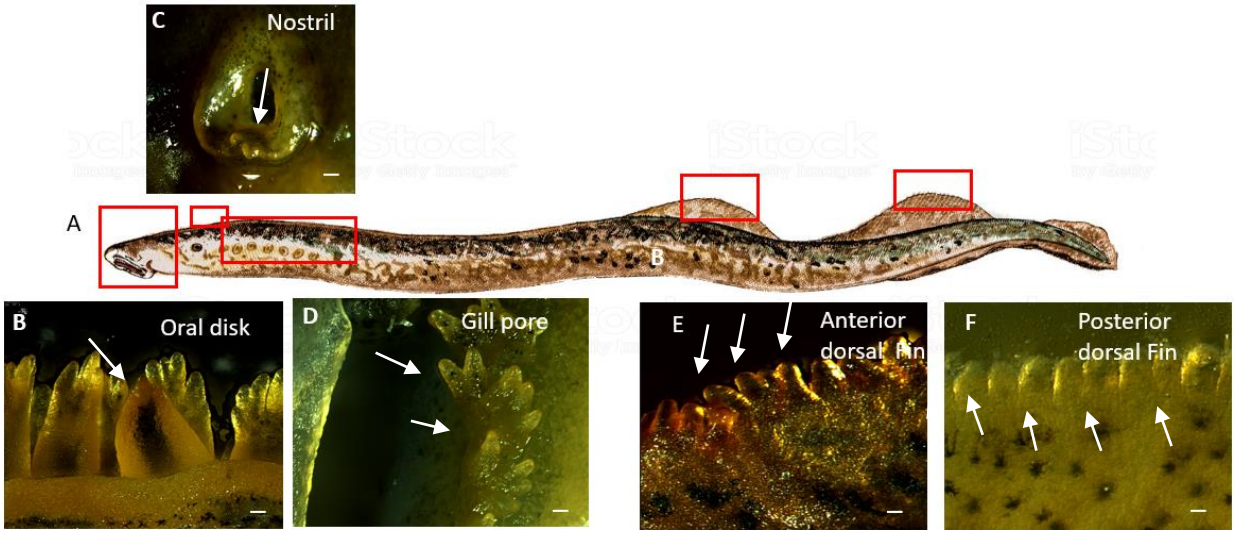

Figure 4.2: Stereomicroscopy of dermal papillae in adult sea lamprey (from B to D) (A) Red boxes indicate the location of the oral disc, nostril, the gills, and the anterior & posterior fins papillae. The white arrows point directly to the papillae. (B) Papillae are located at the outer boundary of the oral disc. (C) Very small papillae are located on the outer boundary of the nostril. (D) Papillae are located at the caudal surface of the gills. (E & F) Abundant papillae were located at posterior edge of the two caudal fins. Scale Bar: B, D and F are 100 µm, E is 75 µm, C is 50 µm.

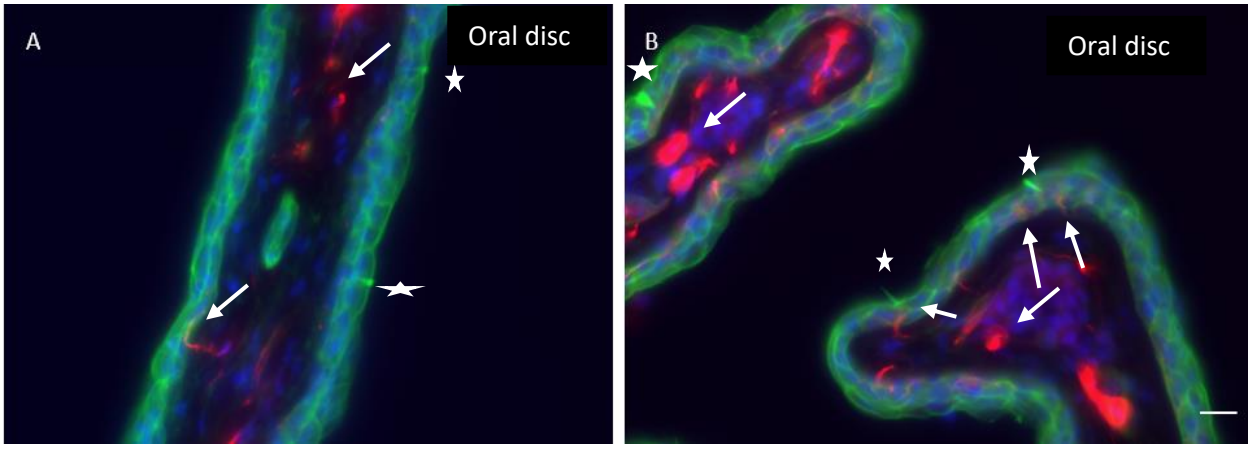

Figure 4.3: Phalloidin and acetylated tubulin labelling of papillae in larval oral disc

The white star indicates the position of phalloidin labelled microvilli. Figure A and B show numerous nerve fibers (white arrows) are found in the lamina propria of basal region of the occasionally enter the epithelium, reaching the basolateral side of SCC cells. Scale bar 50μm.

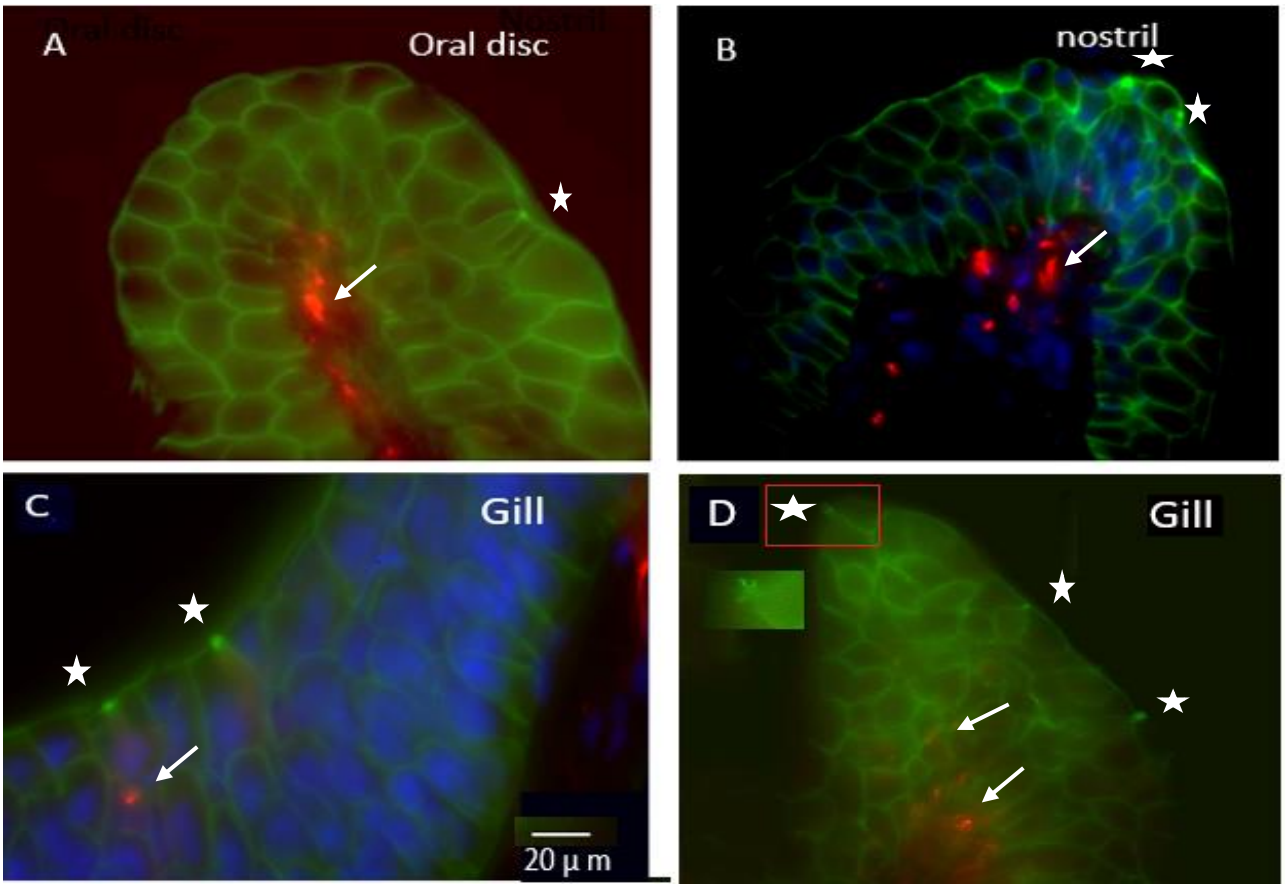

Figure 4.4: Microvillar cells and nerve fibers in transformer stage dermal papillae

Phalloidin (green label, white star) and acetylated tubulin-immunolabeling (red labeling, white arrow) in transformer sea lamprey. Arrow indicates the nerve fibers (red)enter the epithelium and reach close to the basolateral side of microvillar cells in transformer gills, only. In oral disc and nostril, nerve fibers are found at lamina propria. DAPI stains the nuclei blue. Scale bar is 20 µm.

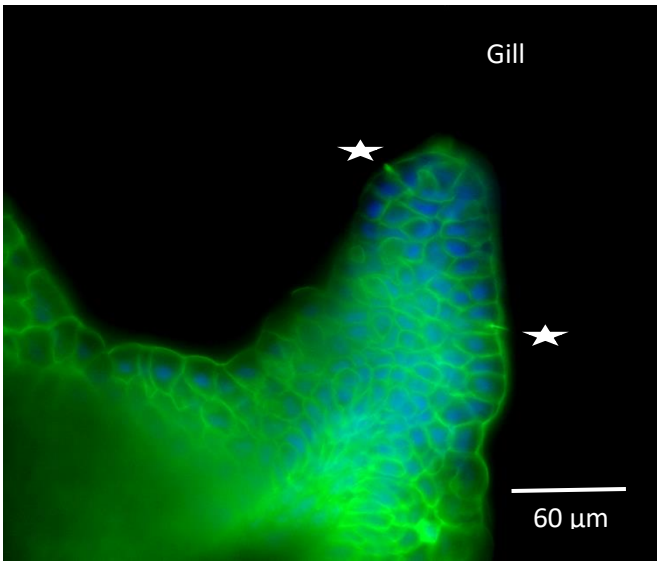

Figure 4.5: Phalloidin labeling on gill papillae of parasitic sea lamprey

Fluorescent phalloidin (green) labels small putative microvilli cells (star) on the gill of parasitic sea lamprey. DAPI stains the nuclei blue. The scale bar is 60 µm.

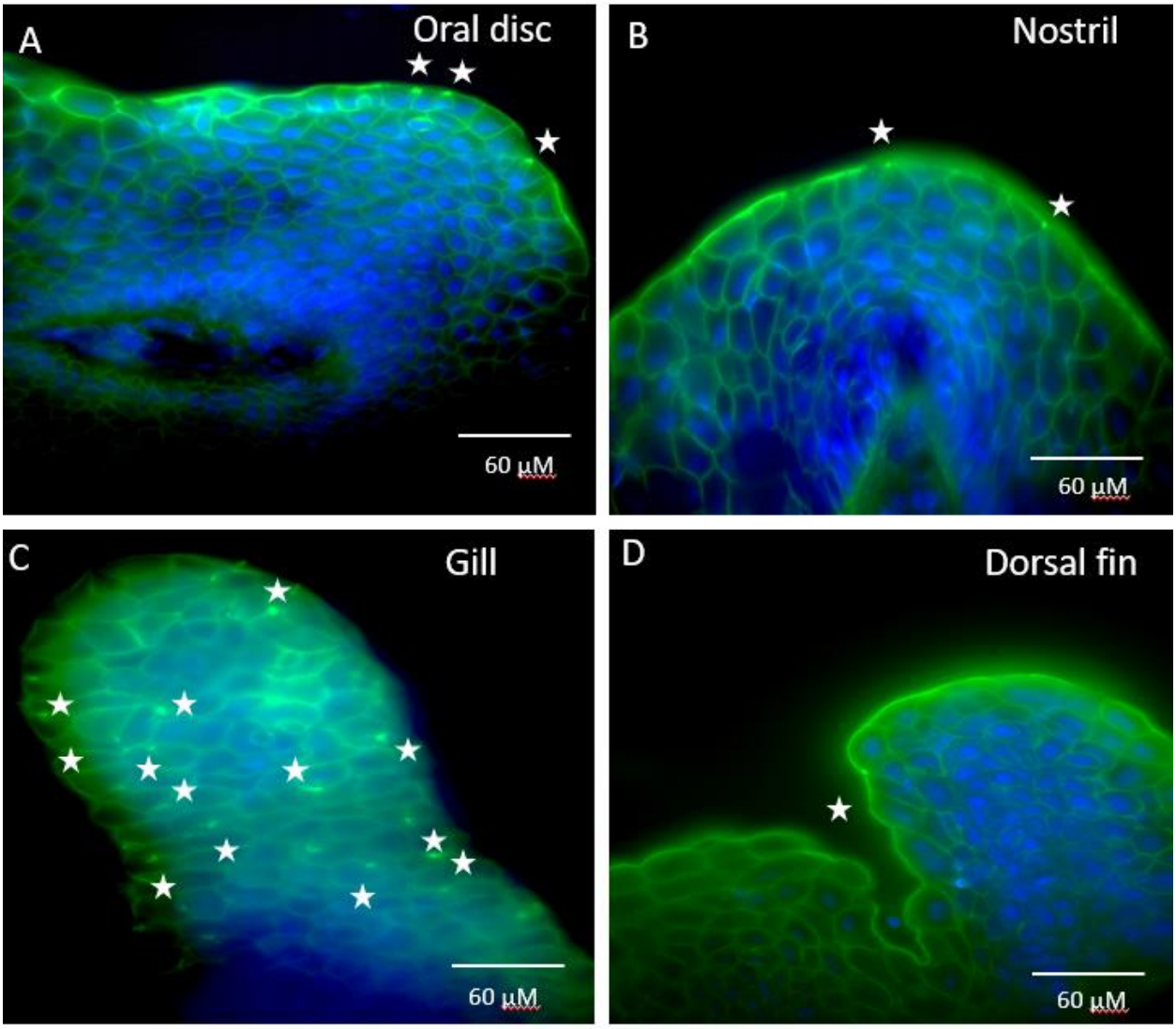

Figure 4.6: Phalloidin labeling on papillae in migratory adult lamprey

Fluorescent phalloidin (green) labels small putative microvilli (star), (A) the oral disc, B) the nostril (C) the gills and (D) the dorsal fin. DAPI stains the nuclei blue. The scale bar in figures A through D represents 20 µm.

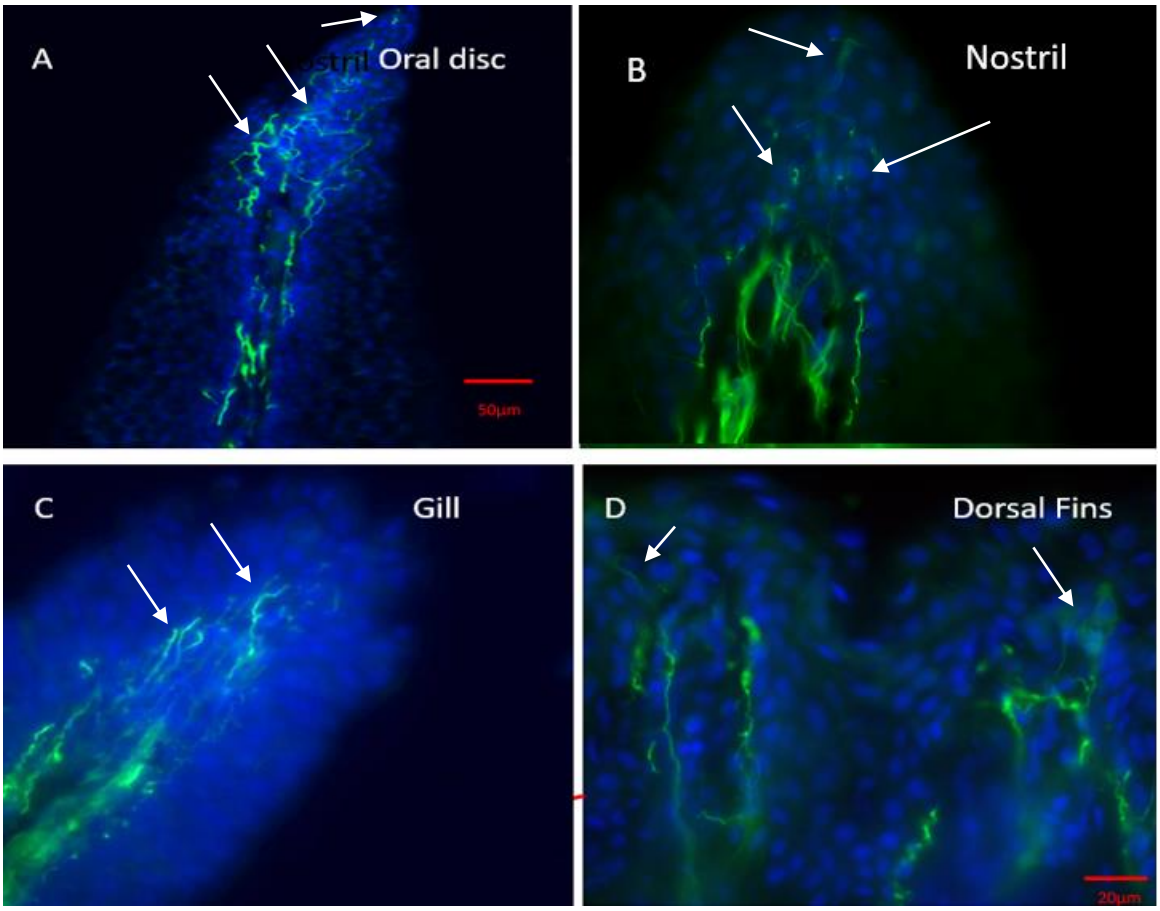

Figure 4.7: Anti-acetylated tubulin labelling of nerve fibers in the papillae of migratory adult sea lamprey

All four regions A) oral disc B) nostril C) gills and D) dorsal fins contained nerve fibers (green labelling). Nerve fibers are abundant in lamina propria below the epithelium. Some nerve fibers enter the epithelium of papillae (white arrows). Blue is DAPI nuclear labelling. Scale bar in A is 50 µm is and 20 µm in B, C and D.

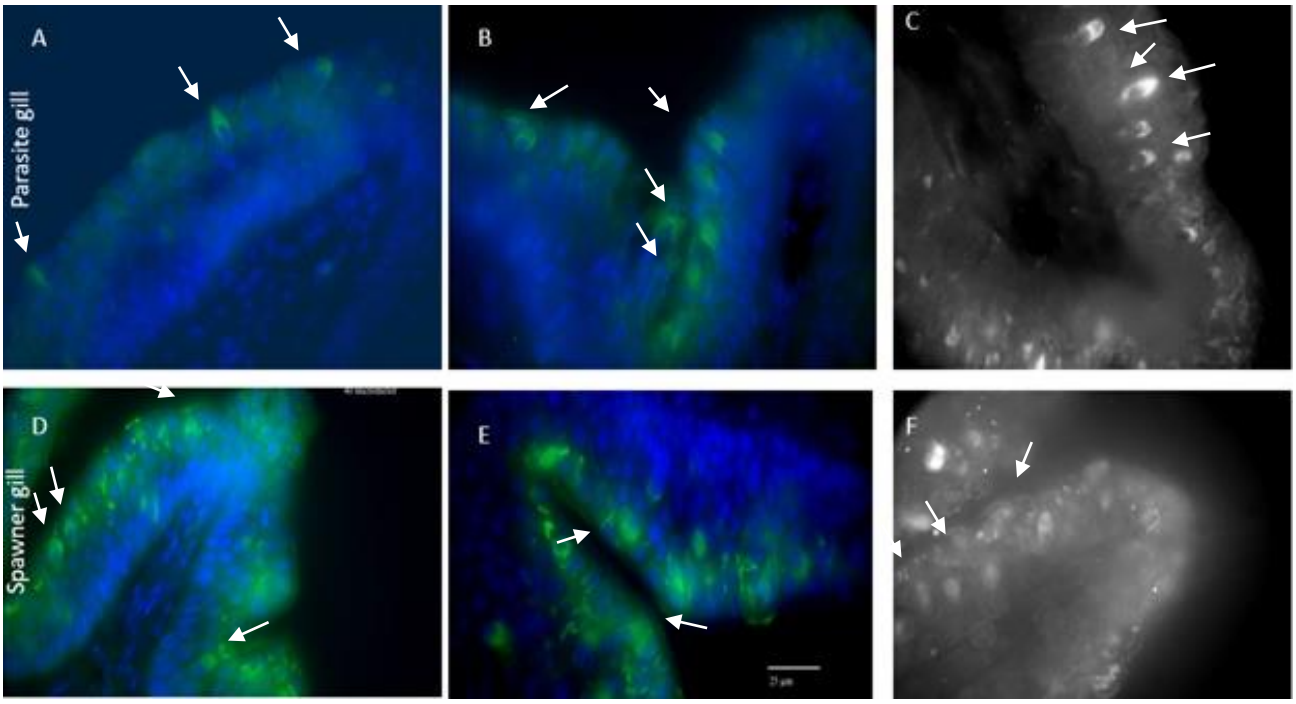

Figure 4.8: Calretinin-immunolabeling in the gills of parasitic and adult sea lamprey

The arrows point to calretinin-immunolabeled SCC cells in the parasitic phase (A to C) and the spawning adult phase. Blue shows DAPI labelling. C and F show calretinin immunolabeling. Scale bar is 60 μm of all the figures.

Table 3: The labeling of SCCs on dermal papillae in different stages and regions on sea lamprey

(–) indicates unsuccessful labelling, a and blank space indicates the tissue was not tested with phalloidin or anti-calretinin.MV indicates phalloidin labeled actin filaments in microvilli and CR indicates calretinin labeling was successful. (-) means absent; (+) means present

(MV- Microvilli), (CR- Calretinin), (SCC- Solitary Chemosensory Cells)

Region	**Larva**	**Transformer**	**Parasite n=3)**	**Spawner (n=6)**
Oral disc	MV on papillae CR (-),n=6	MV on papillae	Not tested	MV on papillae CR (-), n=3
Nostril	No papillae	MV on papillae	Not tested?	MV on papillae CR (-), n=4
Gill	No papillae	MV on papillae CR (-) n=3	MV on papillae CR (+) n=4	MV on papillae CR (+), n=6
Dorsal Fin	No papillae	No Papillae	Not tested	MV on papillae CR (-), n=3

Chapter 5: Thesis summary

5.1 Summary

The objective of my study was to gain an understanding of the function of specific cells within lamprey chemosensory structures: the main olfactory epithelium, the AOO and the dermal papillae that contain the SCCs.

In chapter 2, the study indicated that spermine and spermidine stimulate individual olfactory sensory neurons in the main olfactory epithelium. This calcium imaging experiment was done on 27 olfactory larval epithelial preparation, and 188 cells responded to a specific odorant. Individual cells responded to these polyamines, TCA, amino acids and pheromones including 3kPZS when presented in a mixture and when applied individually. The neutral amino acid L-serine, which is not an odorant failed to stimulate a cellular response. The application of potent odorants in a low Ca^{+2} Ringer solution also failed to elicit a response. These control experiments tell that the cellular olfactory responses due to the binding of the odorant to the receptor that triggers the entrance of Ca^{+2} ions through calcium channel. Moreover, in the lamprey, individual basic amino acids, taurocholic acid, bile acid pheromones, and polyamine odorants stimulated individual olfactory epithelial cells. The separate cell for each odorant response profile may be an essential factor for each odorant, stimulating a specific behavioural response. Moreover, this study also indicated that the olfactory system of sea lamprey was very susceptible to polyamine presence in the surrounding environment. It can detect concentrations as low as 10^{-15}M spermine. Spermidine is less potent than spermine at the concentration of 10^{-11} M. This high sensitivity supports previous behavioural studies of female nesting activity and physiological tests at the tissue level (Scott et al., 2019). Spermine and spermidine may act as “sex pheromones” in sea lamprey. Spermidine and spermine both bind to the different receptors and may elicit different

behaviour response in the sea lamprey. The ovulated female usually prefers sperminated washing of male sea lamprey than a single fractionated component (Johnson et al., 2008). The discovery of other class of odorants, polyamines and their relative attractiveness which produce specific behaviour, can provide platform to use combination of reproduction odorants to manipulate behaviour in sea lamprey and to protect and manage the population of sea lamprey in Great lakes. The understanding how the olfactory system of basal vertebrate, sea lamprey, worked, may provide the glimpse of evolutionary history of vertebrate olfactory system in terms of organization and function. In the future, it would be interesting to investigate if all pheromones and other polyamines, such as putrescine and cadaverine in both higher and lower concentration levels show the same specificity to the cells in the main olfactory epithelium. It has been observed in a mammalian system that the neighbouring OSNs start recruiting if the concentration of odorant increases (Mori et al., 1995; Hildebrand et al., 1997; Malnic et al., 1999). It could also verify that the olfactory sensory neurons within main olfactory epithelium are highly specific to each odorant. It is also important to find the pathway these odorants used in sea lamprey. It was found by Grande, (2017) that the pheromones used cAMP pathway using Forskolin, cAMP activator in sea lamprey. In Zebrafish and Goldfish, the polyamines do not used cAMP pathway. Finding of transduction pathway may also provide new insights into the organization olfactory system of sea lamprey.

While olfactory sensory responses have been observed by recording from the brain region that receives the AOO axons (Green et al., 2017), there still have been no direct observations of AOO cellular responses to odorants. This leaves the exact function, including the cellular response profile of the AOO cells as an unanswered question. The AOO region is significant, as it gates locomotor response to olfaction (Derjean et al., 2010). An understanding of the sensory neurons

in the AOO is vital for understanding lamprey biology, and more broadly, for the knowledge of vertebrate olfactory function and evolution. In chapter 3, the loading of dye was attempted in the cells of the AOO to do a calcium imaging experiment. The cells of the AOO in the larval stage loaded with dye by electroporation occasionally. In transformers, the sensory neurons in the AOO did not load with or without odorant or electroporation. The fluorescent dextran did not enter the cells of the AOO but stopped at the entrance of the duct. In parasite and spawner phase sea lampreys, the fluorescent dextran occasionally entered the lumen and the cells of the AOO following electroporation. The duct and the carbohydrate-containing material may be blocking the entrance of dextran into the AOO cells. This duct may function as a regulator to deliver odorant into the lumen of the AOO. VanDenBossche et al., (1997) stated that blood vessels and nonmyelinated nerve bundles surrounded the AOO. In future experiments, pharmacological application of vasoconstriction and vasodilation to control the blood flow in the blood vessels, surrounding the AOO may result in controlling the flow of fluid in and out of the duct in the sea lamprey. In addition, we can used other dye such as fluro4AM to load the cell of AOO. Fluro-4AM was used in measuring dopamine effect on odor response in mammalian olfactory receptor (Hegg & Lucera, 2004) and monitoring calcium ion dynamic in hair cell (Spinelli et al., 2012). Overall, it the study indicated that the odorant delivery pathway of the AOO is anatomically different from the main olfactory epithelium.

The innervation of SCCs on dermal papillae in different life stages was investigated in chapter 4. The SCCs are spindle-shaped cells with apical microvilli. These cells are present on papillae of the oral disc of the larval stage; and the oral disc, the gill and the nostril after metamorphic transformation. The epithelium of the dermal papilla of each region (oral disc, nostrils, gills, and fins) are innervated with nerve fibres. The presence of the nerve fibres adjacent to SCCs

indicated that the sensory input by the SCCs is likely being transmitted through the nerve fibres, on to higher brain structures. The function of SCCs cells in spawner and parasite is tested by an electrophysiological companion study (Muhammad, in progress). The study indicated that the presence of SCC in different regions suggests that the SCCs may be playing various roles in determining sea lamprey survival and reproduction. The presence of SCC at different phases of sea lamprey may provide insight into its development and major transition from larva to metamorphism stage. The SCC on gills of parasite had been successfully labelled with an antibody against the calcium-binding protein calretinin, suggesting that SCCs are functioning as sensory cells. In the future, it would be interesting to study the physiological and behavioural responses to stimulatory substances of all phase's sea lamprey and inquire about the transduction pathway of SCCs. By learning the SCCs' neuroanatomy and biochemistry and the transduction pathway, we could learn how the SCCs on sea lamprey closed to the SCCs of higher order vertebrate. We could also learn about the evolution history of SCCs by comparing neuroanatomy and biochemistry of SCCs on different species. Overall, the study indicates that the importance of each chemosensory system, their possible role, and how each system work.

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Appendixes

Appendix 1**:** Procedures

A 1 - Calcium imaging (Adopted from Grande, 2017)

1. Switch camera cord to proper computer tower (tape indicates port to insert cord)

2. Turn on Nikon power supply

3. Turn on X-Cite power supply

i. Wait for it to stop flashing (NB: if it is shut off; requires a 1 h cool down period

4. Switch lens filtery to DIA (blank)

5. Turn on chiller; should be set to 6 ºC (NB: requires ~30 mins to reach temp)

6. Turn on ADI power supply (next to Nikon

7. Grab water-immersion objective from drawer

8. On computer:

i. Open Lab Chart (set 10s intervals / pulse duration 5s / 1V amplitude)

ii. Turn on camera (switch at back, on left)

iii. Open Metafluor:

1. Click begin new experiment
2. Under “status”; click “log data”

a. Save under “Calcium Imaging” (folder already in existence)

b. Title experiment as today’s data plus your initials

c. Within folder; make a folder for each odorant tested

3. Repeat a-c for “Save images” and “Save ratios”

iv. Click focus

v. Pull slider (on right side of eyepiece)

vi. Click “Start focusing” ◊ make necessary adjustments to expose and gai

vii. Click “Stop focusing”

viii. “Cfg acq” ◊ set exposure time to what it is in “Focus”

ix. “Acq one”

x. Click “Regions”

    1. Click okay
    2. Click on “O”
    3. Circle each corner of field of view + a few cells
    4. Click save

xi. Click “start” on Lab Chart

xii. In Metafluor; click “Zero clock”, click “Acquire”, and in Lab Chart; enter comment. * **All 3 steps to be done simultaneously***

xiii. In Lab Chart; Stimulate once (5s) at 100 cycles if the preceding 20 cycles were not noisy. Then stimulate again 200 cycles later

xiv. Things to keep in mind:

    1. The room should be kept as dark as possible
    2. Care should be taken to not bump the microscope or the table on which it sits
    3. Gloves should be worse throughout the experiment to prevent your odours from contaminating the ringer’s etc.

A-2 Acetylated tubulin immunocytochemistry protocol as a general label of neural tissue (Adapted from: Frontini, 2002 (MSc Thesis))

1. Incubate the slides, 10 minutes in Acetone in -20-degree temp in the fridge

2. Rehydrate the slides for 10-15 minutes in 0.1 M PBS at room temperature

3. Incubate in primary antibody: monoclonal mouse anti-acetylated tubulin (6-11B, Sigma)1/1000 in 0.1 M PBS plus 5% goat serum at 4 °C for two-three days

4. Wash the slides three times in 0.1 M PBS for 30 min each

5. Incubate in secondary antibody: Alexafluor 488 anti-mouse IgG (A11001, Life Technologies Inc) diluted 1/250 in 0.1 M PBS for one or two days at 4 °C

6. Wash the slides four times in 0.1M PBS for 30 min each

7. Coverslip with Vectashield Mounting Medium with DAPI to label nuclei

A-3 Calretinin immunocytochemistry protocol (adapted from Shi et al. 1993; Brown et al. 1995)

1. Incubate the slides, 10 minutes in Acetone in -20-degree temp in the fridge

2. Rehydrate the slides for 10-15 minutes in 0.1 M PBS at room temperature

3. Incubate the slides for 25 minutes in the heated antigen retrieval solution (2.9 g of Trisodium citrate plus 0.1 % Tween-20, pH to 6.0 or 7.0 , mixed in deionized water)

4. Incubate with Calretinin polyclonal antibody produced in goat and rabbit (7697, Swant) at 1/1000 in 0.1 M PBS plus 5 % goat serum in keeper at 4°C on a shaker for three days

5. Wash the slides three times in 0.1 M PBS for 30 min each

6. Incubate with goat antirabbit Alexafluor 568 IgG (A11011, Life Technologies Inc) diluted 1/250 in 0.1 M PBS for two days at 4°C in a keeper on a shaker.

7. Wash the slides four times in 0.1M PBS for 30 min each

8. Coverslip with Vectashield Mounting Medium with DAPI to label nuclei

A-4 GSIβ$_4$ immunocytochemistry protocol (Adapted from Beausejour et al. 2019, Green, 2012)

1. Incubate the slides, 10 minutes in Acetone in -20-degree temp in the fridge

2. Rehydrate the slides for 10-15 minutes in 0.1 M PBS at room temperature

3. Incubate with Griffonia simplicifolia lectin1 (GS-1 isolectin B4, Cat log # B-1205, Vector, Burlingame, CA; 10 μg/ml in 0.1 M PBS+ 5 % goat serum, pH 7.4) in keeper at 4°C on a shaker overnight

4. Wash the slides two times in 0.1 M PBS for 30 min each

5. Incubate with DCS avidin-fluorescein for 5 hours or overnight (1: 100 in 0.1 M PBS, Cat log 201, Vector, Burlingame, CA, pH 7.4) at 4°C in a keeper on a shaker.

6. Wash the slides three times in 0.1M PBS for 30 min each

7. Coverslip with Vectashield Mounting Medium with DAPI to label nuclei

A-5 Dextran loading using electroporation (Adapted from Weiss et al., 2019)

1. Anesthetize the desired lamprey in the container that contains the required percentage of MS-222 dissolved in dichloride water at 6 °C temperature.

2. Take out the lamprey and placed it on a dry petri dish. The region of interest (e.g. the nostril of the lamprey) was brought under the field of view of the dissection microscope

3. Dry the moisture inside and outside the nostril using Kim wipe to prevent the fluorescent dye from spreading, gently

4. Using needle and syringe, dry fixable dextran crystal ((D3328, 3000 MW, Lysine Fixable) or crystal mixed with Triton-X, are placed into the nasal cavity.

5. Connect two platinum wire electrodes (e.g. diameter 0.2 mm) to a current creating generator connected in parallel.

6. Insert one electrode into the dye-filled the nasal cavity without injuring the olfactory mucosa.

7. Place the second electrode to the exterior, near the nostril or interior, near the nasal. (20-25 Ampere current for 1 min and 0.5s on-off pulses was applied 20-25 ampere with no pulse current (continues current))

8. Remove the electrodes and transfer the lamprey into the beaker with dechlorinated chilled water, odorized with odorant

9. Transfer lamprey into holding aquaria for at least 48 hours.

Appendix 2 : Solutions

2A-1 4% Paraformaldehyde (PFA) for 500 mL

1. Add PFA to 110 ml deionized water
2. Heat to approximately 55 °C and stir the solution for 10 minutes, then clear by adding the NaOH chips while stirring. Add slowly until the solution is clear when dissolved
3. Cool the solution (on ice if desired) to 10°C
4. Add 140 mL of distilled water more
5. Add 150 ml 0.2 M phosphate buffer to the liquid to bring it to a final volume of 500 ml
6. Check pH using pH strips and adjust it to 7.4

2A-2 0.1 M Phosphate buffered saline (PBS)

1. Dissolve 27.6 g sodium Phosphate monobasic into 1 L of deionized water to make stock A.
2. Dissolve 28.4 g Sodium Phosphate Dibasic into 1 L of deionized water to make stock B
3. To make a 0.2 M Phosphate Buffer:
    a. Mix 190 mL of stock A and 810 mL of stock B

To make 0.1 M phosphate-buffered Saline

a. Add 1000 mL of deionized water to1000 mL of 0.2 M PB
b. Add 16.0 g NaCl and 0.4 g KCl to 1 L of 0.1 M PB
c. pH to 7.4

2A- 3 Triton x-100 recipe:

1. To make 10 ml of 0.1 % Triton- 100x
    a. 0.005844 g NaCl in 9.99 ml of deionized water
    b. 10 uL Triton 100x

* Aliquot into 100 ul and store at -20 °C.

2A-4 Stock solutions

Molecule	**Amount to add**	**Amount of solute**	**Final concentration**
L-arginine	0.01742g	10mL Ringer's	10^{-2} M
L-histidine	0.01552g	10mL Ringer's	10^{-2} M
TCA	0.053768g	10mL Ringer's	10^{-2} M
Spermine	0.01 g	50 ml Ringer's	10^{-3}M
NaOH	10g	50mL DI H20	5 M
NaCl	5.84g	50mL DI H20	2 M

odorant	**Aliquot volume (µL)**	**Into x µL of Rnger's**	**Final concentration**
3kPZS	49	9951	10^{-5} M
Spermidine	3	19997	10^{-3} M
PAMS-24	64.6	9935.4	10^{-5} M

VitaAuctoris

Name: Zeenat Aurangzeb

Place of Birth: Lahore, Pakistan

Year of birth: 1991

Education: B.Sc. Biological Science and Biotechnology,

University of Windsor

2009-2015

M.Sc. Biology Sciences

University of Windsor

2016-2019