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Olfactory biology of two vertebrate species: Central projection of a subpopulation of olfactory sensory neurons in the sea lamprey (Petromyzon marinus) and the production of putative reproductive pheromone(s) in the round goby (Neogobius melanostomus)

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OLFACTORY BIOLOGY OF TWO VERTEBRATE SPECIES: CENTRAL PROJECTION OF A SUBPOPULATION OF OLFACTORY SENSORY NEURONS IN THE SEA LAMPREY (*Petromyzon marinus*) and THE PRODUCTION OF PUTATIVE REPRODUCTIVE PHEROMONE(S) IN THE ROUND GOBY (*Neogobius melanostomus*)

by

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Abstract

Both the sea lamprey (*Petromyzon marinus*) and the round goby (*Neogobius melanostomus*) are two fish species that invaded the Great Lakes; they likely use sex pheromones that are fundamental to their reproductive success. Understanding pheromone communication could potentially lead to population control of these two species.

In the larval sea lamprey, topographic and spatial projections from the olfactory sensory neurons (OSNs) to the olfactory bulb were studied. These projection patterns may be an initial step in organizing olfactory odourant information. The differential expression of G proteins forms spatial sub-regions consisting of OSN subsets in the olfactory epithelium projecting to discrete glomeruli. In the lamprey, all glomeruli are immunoreactive (IR) for the GTP binding protein G_{olf}, except the medial glomeruli. Following micro-injection of fluorescent dextran into these G_{olf}- non-IR medial glomeruli, back-filled G_{olf}-non-IR OSNs were present in the ventral hemisphere of the peripheral olfactory organ. In contrast, G_{olf}-IR OSNs projecting to non-medial G_{olf}-IR glomeruli were widely distributed in the olfactory epithelium. This suggests the existence of a spatially distinct olfactory sensory pathway in the sea lamprey that is reminiscent of olfactory systems in other vertebrates.

Studies in the round goby indicate that the reproductively mature male releases 5β-reduced androgenic steroidal pheromone(s) that attracts ripe females. Steroid biosynthesis of these compounds was studied in both the testes and the seminal vesicles. The testis contains specialized glandular tissue that may be responsible for the production of pheromonal 5β-reduced androgens. The seminal vesicle may produce and store pheromonal steroids; excretions from this organ may serve as a vehicle for pheromones

to enter the aqueous environment. *In vitro,* the testes converted [^{3}H]-androstenedione into: 3α-hydroxy-5β-androstane-11,17-dione (11-oxo-etiocholanolone, 11-oxo-ETIO); 11-oxo-ETIO sulfate (11-oxo-ETIO-s); 11-oxo-testosterone (11-ketotestosterone), 3α-hydroxy-5β-androstan-17-one (etiocholanolone, ETIO); 11β-hydroxy-androstenedione; ETIO sulfate and testosterone. *In vitro*, the seminal vesicle converted [^{3}H]-androstenedione into: 11-oxo-androstenedione, 11-oxo-testosterone, and 11-oxo-etiocholanolone. Neither 11-oxo-ETIO nor 11-oxo-ETIO-s have been previously identified in teleost gonads. Both 11-oxo-ETIO and 11-oxo-ETIO-s are putative pheromones in the round goby, given that the carbon A ring has a 5β-configuration that has been linked with olfactory sensitivity and behavior induction in two other species of gobies.

Co-authorship statement

I certify that this thesis and research are original products of my research, and that ideas and quotations from the work of others, published or otherwise are fully acknowledged in accordance with the referencing practices of the discipline. I acknowledge the valuable contributions of the following in Chapter 3: Andrea J. Bélanger, Lynda D. Corkum, Barbara S. Zielinski, Weiming Li, Sang-Seon Yun and Alexander P. Scott.

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List of Abbreviations

AOB	Accessory Olfactory Bulb
ATP	Adenosine Triphosphate
cAMP	cyclic Adenosine Monophosphate
EOG	Electro-olfactogram
GDP	Guanosine Diphosphate
GTP	Guanosine Triphosphate
G_{olf}-IR	G_{olf}-Immunoreactive
G_{olf}-non-IR	G_{olf}-non-Immunoreactive
HPLC	High-Performance Liquid Chromatography
IP_3	Inositol Triphosphate
-IR	Immunoreactivity
MOB	Main Olfactory Bulb
MS222	Tricaine methane sulfonate
OB	Olfactory bulb
OE	Olfactory epithelium
ON	Olfactory nerve
OSN(s)	Olfactory sensory neuron(s)
PBS	Phosphate buffer saline
TLC	Thin Layer Chromatography
VNO	Vomeronasal organ

Chapter 1: General Introduction

1.1 Thesis Overview and Significance

In these two individual studies, model systems were implemented whereby both the detection of pheromones through olfaction and production of pheromones could be studied in vertebrate systems. Pheromone communication appears to be important for the survival and population expansion of the ancestral jawless-agnathan, the sea lamprey (Li et al., 2002) and for the round goby, a jawed-gnasthotome (Murphy et al., 2001). These two fish species have invaded the Great Lakes (Corkum, et al., 1998; Li et al., 2002).

In Chapter 2, the organization of the olfactory system in the sea lamprey (*Petromyzon marinus*) is assessed at the level of the olfactory bulbs and the peripheral olfactory organ. Unique pathways of neurons were studied that connect the olfactory bulb and the olfactory epithelium. In this study, a spatially distinct olfactory sensory pathway is identified that could possibly function in the detection of pheromones or distinct odorants.

In Chapters 3 and 4, the identification of putative reproductive sex pheromones in the round goby was established. *In vitro* incubations of the male gonad provided insight into the putative reproductive pheromones released by the male that attract the female to its nest during spawning. The pheromones identified could be implemented in species control. In this study, putative pheromones have been isolated, and further studies are required to confirm their function.

1.2 The primary olfactory pathway

The olfactory system is a well-developed sensory system that is responsible for discriminating and detecting numerous low molecular mass odourant molecules (reviewed by Firestein, 2001). In order to accomplish this, the olfactory system possesses mechanisms to discriminate among the array of odourants it may confront in its environment. Olfactory research has indicated that this sensory system is essential in order for animals to find food, detect predators and prey and in mate detection.

From agnathans to primates, the organization of the vertebrate olfactory system and the primary olfactory pathway is fundamentally similar. The primary olfactory pathway consists of olfactory sensory neurons (OSNs) that cover the olfactory epithelium. These OSNs converge together to form the olfactory nerve (cranial nerve I) that projects onto the olfactory bulb. OSNs located in the olfactory epithelium are part of the peripheral nervous system. OSNs are bipolar neurons, with an apical dendrite located in the olfactory epithelium which project to the olfactory bulb. The apical dendrite of the OSN extends either cilia or micrivilli onto the mucosal surface of the olfactory epithelium, which is the site of signal transduction. The projection of numerous OSNs to particular neuropil in the olfactory bulb forms the olfactory bulb glomeruli. The olfactory bulb glomeruli are spherical, typically 50-100 μm in diameter, and contain OSN terminals. Here the axons of OSNs project onto mitral cells, which in turn carry information to higher brain centers (Figure 1). In fish, projection of second-order neurons (mitral cells) to the telencephelon forms the olfactory tract. In teleosts, the olfactory tract is divided into medial and lateral components. The medial olfactory tract is composed of fibres projecting from the medial olfactory bulb; the lateral olfactory tract

is composed of fibres projecting from the lateral olfactory bulb (Laberge and Hara, 2001). In many terrestrial vertebrates, a separate chemoreceptive structure is also present, the vomeronasal organ (VNO). The epithelium of the VNO contains its own OSNs that form the vomeronasal nerve that projects to glomeruli in the accessory olfactory bulb (AOB) (Halpern, 1987).

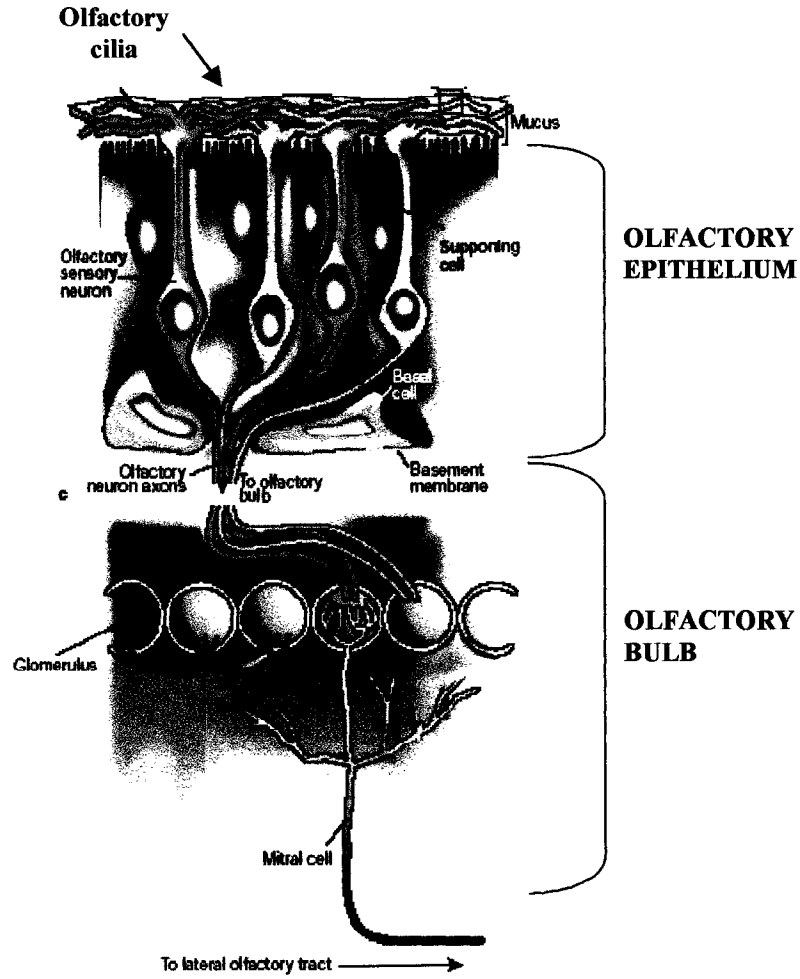

Figure 1. The organization of the primary olfactory pathway. The primary olfactory pathway consists of olfactory sensory neurons (OSNs) that cover the olfactory epithelium which converge together to form the olfactory nerve that projects onto the olfactory bulb. OSNs as well as supporting sustentacular cells comprise the olfactory epithelium. The OSNs converge to form the olfactory nerve and project to the olfactory bulb glomeruli where they synapse onto mitral cells. In mammals, it has been shown that axons from all OSNs expressing a particular receptor converge onto the same glomerular unit. In mammals, mitral cells leaving the olfactory bulb project to higher brain centers; in fish, mitral cells project to the telencephelon. The projections of mitral cells forms the olfactory tract (Adapted from Firestein, 2001).

1.3 Sensory transduction of olfactory information

Olfaction is an attractive sense to study since the organization of signaling pathways in olfactory systems appears to be evolutionarily conserved (Hildebrand and Shepherd, 1997). Signal transduction occurs within the membrane of the OSN, and commences when a receptor binds an odourant molecule. G proteins (located on the intracellular surface of the cell membrane) are linked to receptor proteins that extend to the exterior surface of the cell. GTP-binding proteins (G proteins) are heterotrimers ($\alpha\beta\gamma$) that couple the OSN membrane-bound receptor to second-messenger enzymes or ion channels (Jones and Reed, 1989). The α subunit gives the identity to the heterotrimer; the α subunit governs the specificity of the interaction with receptor and effector molecules such as second messengers (Jones and Reed, 1989). G proteins in the OSN use second messengers such as cyclic AMP (cAMP), and inositol triphosphate (IP_3) to affect the activity of ion channels. Consequently, there are many different subtypes of the α subunit expressed in OSNs. For example, $G_{\alpha olf}$ is expressed in mammalian (Jones and Reed, 1989) and fish (Bélanger et al., 2003; Frontini et al., 2003; Hansen et al, 2003) OSNs. As well, the expression of $G_{\alpha q}$, $G_{\alpha 11}$ in OSNs has also been indicated in both mammals and fish (Dellacorte et al., 1996).

The G protein, $G_{\alpha olf}$ is an olfactory specific subtype shown to be present in agnathans (Frontini et al., 2003), teleosts (Hansen et al, 2003), amphibians (Mezler et al., 2001) and mammals (Jones and Reed, 1989). It is cAMP dependent and linked to olfactory receptors which mediate olfactory signaling (Jones and Reed, 1989). The signal transduction cascade commences when an extracellular odourant molecule binds the receptor protein on the surface of the OSN, which activates the G protein $G_{\alpha olf}$ (Jones and

Reed, 1989). In turn, $G_{\alpha olf}$ activates adenylyl cyclase (Pace, 1986). The cyclase converts the intracellular ATP into cAMP, a molecule that has numerous signalling roles in cells. The cAMP binds to the intracellular face of an ion channel, allowing the conduction of Na^+ and Ca^{+2} into the cell (Firestein et al., 1991) (Figure 2, adapted from Firestein, 2001). Inactive OSNs maintain a resting voltage across their plasma membrane of about –65mV (inside with respect to the outside). The influx of Na^+ and Ca^{+2} ions causes the inside of the cell to become less negative compared to the outside. If enough ion channels remain open for an extended period of time, and the membrane potential becomes about 20mV less negative than the outside, the cell reaches threshold and generates an action potential in the axon hillock. The action potential propogates along the axon of the OSN until it meets the axon terminal and forms a synaptic junction with second order neurons (such as mitral cells) in the olfactory bulb. Hence, the cAMP pathway is imperative for the functioning of OSNs. Inositol triphosphate (IP_3) is another second messenger system that has been implicated in signal transduction in OSNs (Restrepo et al., 1993). It has been shown that different G proteins regulate different second messenger systems and that selected second messengers are activated by specific odourants (Breer and Boekhoff, 1991). For example, floral and putrid odourants selectively activate the cAMP and IP_3 second messenger systems, respectively (Breer and Boekhoff, 1991). Responses to most odourants probably involves both second messenger systems whereby calcium mediates crosstalk between these two major olfactory signal transduction cascades (Anholt, 1993). Hence, the study of the expression of G proteins within the olfactory system can provide much insight into olfactory coding mechanisms.

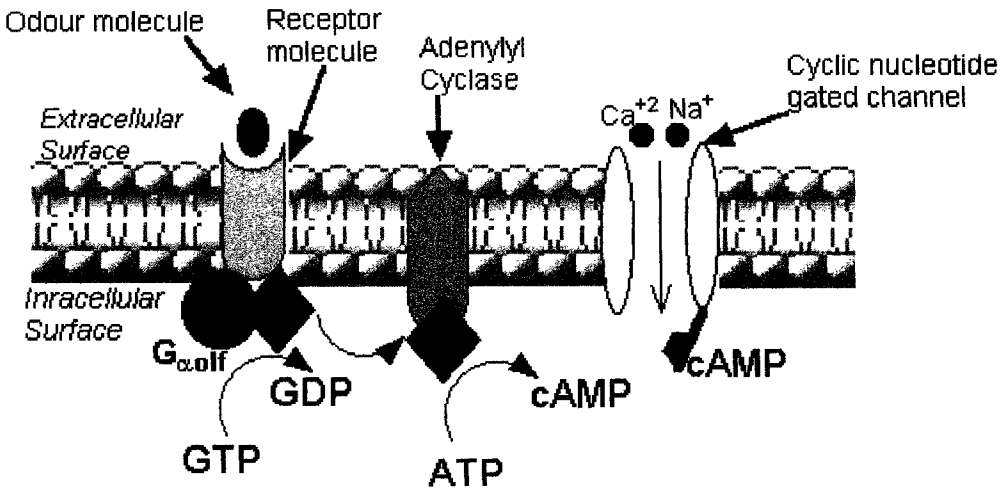

Figure 2. Sensory transduction within the membrane of an olfactory sensory neuron. The Gα subtype, $G_{\alpha olf}$, is linked to the cAMP second messenger. An odourant molecule binds to the receptor molecule and the receptor-odorant complex activates a G-protein ($G_{\alpha olf}$), causing binding to a GTP molecule, which releases a molecule of GDP. The activated G-protein dissociates and activates a cyclase which converts the intracellular ATP into cyclic AMP (cAMP), a molecule that has numerous signaling roles in cells. The cAMP binds to the intracellular face of an ion channel, allowing the conduction of Na^+ and Ca^{+2} into the cell (Adapted from Firestein, 2001).

1.4.1 The organization of the olfactory system

The primary olfactory pathway consists of the OSNs in the olfactory epithelium which project to the olfactory bulb glomeruli. Olfactory bulb glomeruli are formed by the convergence of OSN axons from the olfactory nerve. Studies in fish olfaction have indicated that the processing of odourant information begins in the olfactory epithelium and continues in the olfactory bulb and higher brain centers (see the discussion that follows).

1.4.2 The organization of the olfactory system: the use of G-proteins

Throughout evolution, G-proteins have been implemented in organizing olfactory information in the olfactory system (Hansen et al., 2003; Jia and Halpern, 1996; Juilfs et al., 1997; Frontini et al., 2003). From early craniates such as the jawless fishes (lampreys and hagfishes), which evolved over 400 million years ago, to mammals which evolved 190 million years ago, the use of G proteins in the olfactory system seems to be a common element. The expression of various G-proteins in subsets of OSNs in the olfactory epithelium appears to be fundamental for odourant discrimination in the olfactory system of vertebrates (Frontini et al., 2003; Jia and Halpern, 1996; Hansen et al., 2003). The differential expression of G proteins in the olfactory system indicates the use of alternate signal transduction mechanisms in odourant detection. Since the lamprey evolved much before mammals and many other vertebrates, these living jawless fishes provide a window of insight into the earliest vertebrate olfactory systems. In the sea lamprey, which is a living representative of early vertebrate evolution, of the 7 glomerular territores present, one glomerular territory, the medial glomeruli, does not express the G protein $G_{\alpha olf}$ (Frontini et al., 2003). This indicates that alternate olfactory signal transduction cascades may be implemented in early craniate radiations. Further evidence of the use of alternate G proteins in the olfactory system exists in the gnathostomes (jawed vertebrates). In the catfish (*Ictalurus punctatus*), a jawed vertebrate, the expression of the Gα subtypes, $G_{\alpha o}$, $G_{\alpha q/11}$ and $G_{\alpha olf}$ is present in segregated regions of the primary olfactory pathway (Hansen et al., 2003). In the *Xenopus laevis*, an amphibian, two different Gα subtypes are expressed in the olfactory epithelium: $G_{\alpha o1}$ in the lateral diverticulum and $G_{\alpha s}$ in the medial diverticulum (Mezler et

al., 2001). In the mammalian vomeronasal organ, receptor neurons that express the G proteins $G_{i\alpha 2}$ and $G_{o\alpha}$ project to segregated glomeruli within the accesory olfactory bulb (Jia and Halpern, 1996). Taken together, the differential expression of G proteins and the use of alternate signal transduction mechanisms seems to be an underlying principle in the olfactory system which developed in ancient vertebrate ancestors in the agnathan 400 million years ago.

1.4.3 The organization of the olfactory system: spatial projections of OSN terminals from the olfactory epithelium in the nose to the olfactory bulb.

Many studies in vertebrates have been conducted to determine the importance of topographic projections between the olfactory epithelium and the olfactory bulb. In the hamster, the presence of spatial projections of OSNs in the olfactory epithelium onto olfactory bulb glomeruli was detected through micro-injections into specific olfactory bulb glomeruli and subsequent back-filling of OSNs in the olfactory epithelium. It was concluded that OSNs in specific zones of the olfactory epithelium project onto specific areas of the olfactory bulb (Schoenfeld et al., 1994). This zone-to-zone "spatial topographic" mapping has been termed rhinotopy (Schoenfeld et al., 1994). The presence of a zonal map in mammals has been confirmed through determining the expression of specific olfactory receptor genes in OSNs. It has been shown that OSNs that express a specific olfactory receptor gene is expressed within one of four zones in the olfactory epithelium (Ressler et al., 1993; Vassar et al., 1993); furthermore, OSNs in each zone project to specific glomeruli in the olfactory bulb (Mori et al., 1999). Hence, OSNs that express a certain receptor converge onto distinct glomeruli (Mombaerts, 1999; Mori et al., 1999). Therefore, in mammals, a zonal map is formed whereby specific

areas of the olfactory epithelium (containing OSNs that express distinct olfactory receptor genes) projects to distinct glomeruli. This implies that OSN afferents segregate by odourant responsiveness and that local regions of the olfactory bulb receive input from OSNs with similar odourant response properties (Mori et al., 1999) (Figure 3).

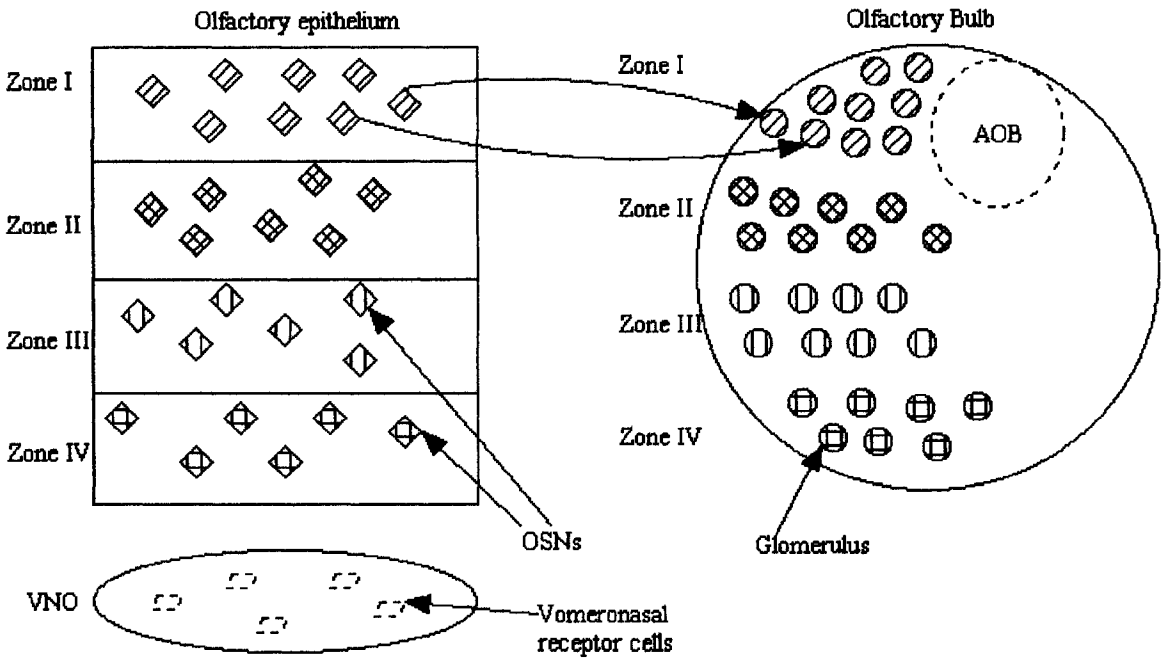

Figure 3. Schematic diagram showing the axonal connections between the olfactory epithelium in the nose and the main olfactory bulb (MOB). The accessory olfactory bulb (AOB) receives axonal inputs from the vomeronasal organ (VNO). The VNO contains its own vomeronasal receptor cells and is separate from the olfactory epithelium. In mice, extensive studies have indicated that the OE is divided into four zones, based on the expression of odourant receptors. OSNs in a specific zone of the OE project to glomeruli located in a corresponding zone of the MOB. Axons of OSNs expressing the same odourant receptor converge to defined glomeruli. (Adapted from Mori et al., 1999)

Although a strict zone-to-zone pattern of projection between the olfactory epithelium and the olfactory bulb has been established in mammals, not all olfactory systems display this. In fish, the Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss*) and the zebrafish (*Danio rerio*) appear to have a slightly different method of olfactory coding. OSNs with common odourant receptors are randomly

dispersed in the olfactory epithelial sheet and converge onto common glomeruli. In the zebrafish, the existence of distinct zones of the epithelium projecting to distinct zones of the olfactory bulb was not apparent (Baier et al., 1994). Retrograde tract studies indicated that regardless of which olfactory bulb glomeruli were micro-injected with a neuronal tract tracer, back-filled OSNs in the olfactory epithelium were widely dispersed over the olfactory epithelium (Baier et al., 1994). Likewise in the rainbow trout, projections from the olfactory epithelium to the olfactory bulb were non-topographically ordered (Riddle and Oakley, 1991). Anterograde tract tracing in the rainbow trout indicated that regardless of where the dye was applied in the OE, labeled axons were never restricted to a subregion of the glomerular layer (Riddle and Oakley, 1991). Furthermore, retrogradely in the rainbow trout, regardless of which glomerulus was injected, labeled OSNs were widely dispersed in the OE (Riddle and Oakley, 1991).

All the previous species discussed here (section 1.3.3) were all jawed gnathostomes: teleost fish and mammals. In Chapter 2 of this thesis, the objective was to determine whether the spatial projections in the primary olfactory pathway of the sea lamprey, a jawless agnathan, possess diffuse projections like the teleost model or topographically ordered projections like mammals.

1.4.4 The organization of the olfactory system: the spatial division of the olfactory system and neural pathways for chemoreception.

In both fish and mammalian olfactory systems alike, research has indicated the presence of independent neural pathways for the chemoreception of pheromones (Friedrich and Korsching, 1998; Hara and Zhang, 1996; Halpern, 1987; Kyle et al., 1987; Laberge and Hara, 2003; Stacey and Kyle, 1983; Sorensen et al., 1991; Weltzien et al.,

2003). Sex pheromones are defined as "a substance, or mixture of substances, which is released by an individual and which evokes a specific and adaptive reproductive response in conspecifics, the expression of which does not require specific learning" (Sorensen and Stacey, 1999). Mammals possess a separate chemoreceptive structure, the VNO, which is located at the base of the nasal septum of most terrestrial vertebrates (Halpern, 1987). The vomeronasal system consists of a peripheral organ, the VNO, which contains its own sensory neurons that project to the accessory olfactory bulb (AOB). This is separate from the olfactory epithelium, which contains sensory neurons that project to the main olfactory bulb (MOB). The processing of pheromonal information in many mammalian species has been shown to occur in the VNO (Halpern, 1987). The VNO also mediates the detection of non-pheromonal compounds (Halpern, 1987). On the contrary, although fish do not possess a VNO, the vomeronasal system may be present in some fishes in a form that has not yet been recognized (Eisthen, 1992). The functional division of the olfactory system into medial and lateral components in fish may be analogous to the functional division of the VNO and the olfactory system in mammals (Eisthen, 1992; Dulka, 1993). In the goldfish, the medial olfactory tract, responsive to reproductive sex pheromones, may be functionally analogous to the tetrapod VNO; the lateral olfactory tract, responsive to amino acids, may be functionally analogous to the tetrapod main olfactory system (Dulka, 1993).

Similar to the goldfish, many other teleost fish display a similar functional division of the olfactory system (Hansen et al., 2003; Laberge and Hara, 2003; Stacey and Kyle, 1983; Kyle et al., 1987; Sorensen et al., 1991; Friedrich and Korsching, 1998; Hara and Zhang, 1996; Weltzien et al., 2003). In zebrafish (*Danio rerio*), the medial

glomeruli respond to stimulation by bile acids; the medial ventral glomeruli respond to a reproductive protaglandin pheromone; and the anterior and lateral glomeruli respond to amino acids (Friedrich and Korsching, 1998). Likewise, in the catfish *(Ictalurus punctatus)*, the medial region of the olfactory bulb is bile salt receptive, and ventral area is amino acid receptive (Hansen et al., 2003). In the Atlantic salmon (*Salmo salar*) and the rainbow trout *(Ocorhynchus mykiss)*, bile acids stimulate the medial olfactory bulb and amino acids stimulate the lateroposterior olfactory bulb (Hara and Zhang, 1996). When the medial olfactory tract of the crucian carp was cut, reduced reproductive behavior was displayed; this indicates that the medial olfactory tract mediates reproductive behavior (Weltzien et al., 2003). Furthermore, in lake whitefish (*Coregonus clupeaformis*), putative reproductive pheromones stimulated a transition area in the olfactory bulb-telencephelon area (Laberge and Hara, 2003).

Therefore, in both fish and mammals, two phylogenically divergent vertebrates, there are spatially segregated pathways for the detection of discrete odourants in the olfactory system. The possibility exists that the medial olfactory system in fish is functionally homologous to the vomeronasal system of higher vertebrates.

1.5 The lamprey as a model of olfaction

The goal of Chapter 2 was to implement the larval sea lamprey model to study evolutionarily conserved characteristics of the olfactory system. The sea lamprey is a living jawless fish, which represents the only vestiges of a large and diverse group of early craniates which flourished nearly half a billion years ago. Comparing the jawless

fishes with gnathostomes can not only aid in determining which characteristics are primitive, but can provide much insight into the earliest vertebrates (Braun, 1996).

In comparison to the gnasthostome radiation, the sea lamprey offers an inherently simple olfactory system to study. In mammals, 1000 different olfactory receptor genes are expressed by OSNs (Buck and Axel, 1991) and the olfactory bulb contains approximately 2400 glomeruli (Meisami et al, 1993). In teleosts, the number of putative olfactory receptor genes is only about 100 (Ngai, et al., 1993), with approximately 80 glomeruli (Baier and Korsching, 1994; Friedrich and Korsching, 1998). In the lamprey, the family of olfactory receptor genes is considerably smaller than in mammals, and may be even smaller than that of teleost fish (Freitag et al., 1999). Hence, lampreys respond only to a few odourants: basic amino acids and bile acids (Li et al., 1995). The total number of glomeruli in the larval sea lamprey ranges from 41-65 (Frontini et al., 2003). Furthermore, a convenient feature of the lamprey olfactory system is that the entire primary olfactory pathway of OSNs is located in the same horizontal plane (Zaidi et al., 1998). A horizontal section through the primary olfactory pathway of the larval sea lamprey displays dendrites and cell bodies in the olfactory epithelium, axons in the olfactory nerve and olfactory bulb (Figure 4). Due to this apparent simplicity in the larval sea lamprey, it is an excellent model for studying olfaction.

The glomerular territories that exist in the larval sea lamprey have been established (Frontini et al., 2003). The fact that the G protein G_{olf}, is lacking in the medial glomeruli of the larval sea lamprey (Frontini et al., 2003), gives way to determining the role of alternate G proteins in odourant coding. Finally, since a reproductive pheromone has been identified in the sea lamprey (Li et al., 2002), studies

can be conducted to study pheromone activation in the primary olfactory pathway in the agnathan.

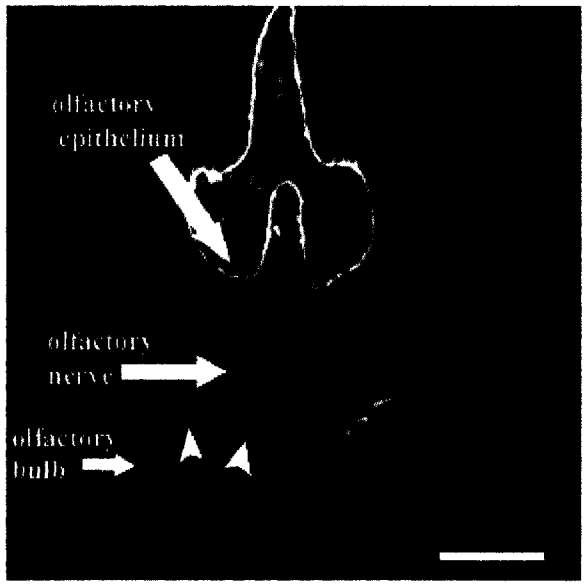

Figure 4. The primary olfactory pathway in the larval sea lamprey. This micrograph shows a primary olfactory pathway after the application of $GS1B_4$ lectin histochemistry. This staining technique highlights the olfactory bulb glomeruli (filled arrowheads) in the olfactory bulb and the surface of the OSNs in the olfactory epithelium.
Scale bar is 0.5 mm.

1.6.1 The use of reproductive pheromones in fish

Fish reside in an aqueous universal solvent in an environment that is dimly lit. The aqueous environment of fishes provides them with a vehicle for transmitting body metabolites. Research has revealed that fish use chemical signals (i.e. pheromones) which mediate reproductive behavior. In fish, the detection of pheromones is mediated through olfaction, which reflects a specialization of this neural system (Sorensen and Stacey, 1999). The study of pheromones can provide much insight into evolutionarily conserved patterns in the vertebrate endocrine system; furthermore, it can provide insights into the functioning of afferents of cranial nerve one (i.e. the olfactory system)

(Stacey and Sorensen, 2002). Fish olfactory systems are responsive to four chemical classes of odourants: amino acids, bile acids, sex steroids and prostaglandins (Zielinski and Hara, 2000). Bile acids, steroids and protaglandins have long been indicated as reproductive pheromones produced in fish (Colombo et al., 1977; Li et al., 2002; Sorensen et al., 1988).

1.6.2 Fish Pheromones: Prostaglandins

Prostaglandins are lipid derived molecules which act as physiological regulators; this includes mediating reproductive functions. Prostaglandins are C_{20} compounds (Figure 5). In the goldfish, F type protaglandins (PGFs) have been shown to be strong olfactory stimulants and are part of the postovulatory female sex pheromone system (Sorensen et al., 1988). The ovulated female goldfish releases PGFs to the water where they are detected by the male olfactory system and stimulate male spawning behaviour (Sorensen et al., 1988). Olfactory epithelial sensitivity to prostaglandins has also been exhibited in the common carp (*Cyptinus carpio*) (Irvine and Sorensen, 1993). (See section 1.7 for the complete discussion of prostaglandin mediation in the goldfish sex pheromone system).

In the lake whitefish, putative pheromonal prostaglandins selectively stimulate a transition area in the olfactory bulb-telencephelon transition area (Laberge and Hara, 2003); in zebrafish, the medial ventral glomeruli respond to a reproductive protaglandin pheromone (Friedrich and Korsching, 1998).

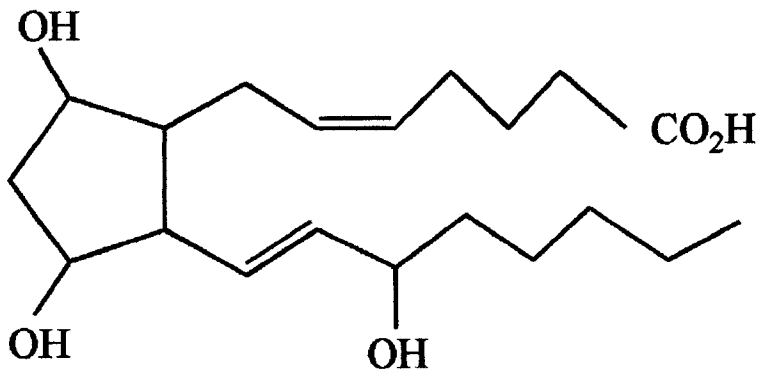

Figure 5. Prostaglandins are C_{20} compounds.
The prostaglandin shown here is PGF2α.

1.6.3 Pheromones: Bile acids

Bile acids are synthesized in the liver from cholesterol and contain the steroid nucleus with a branched side chain of three to nine carbon atoms ending in a *carboxyl group*. Bile acids are amphipathic molecules, having both hydrophobic and hydrophilic portions. Bile acids are C_{24} compounds; various substituents at the R_1 and R_2 positions on the compound yields different bile acids with unique chemical identities (Figure 6). In the mammalian duodenum, bile salts surround lipid droplets, forming an emulsion that aids in digestion. Studies have indicated that bile acids elicit olfactory epithelial responses in teleosts such as the brown trout (*Salmo trutta*), channel catfish, and goldfish (Sorensen and Caprio, 1997, Table 2).

Bile acids act as pheromonal compounds in the jawless sea lamprey (Li et al., 2002; Siefkes et al., 2003). In the sea lamprey, the bile acid 7α,12α,24-trihydroxy-5α-cholan-3-one 24-sulfate, has been implicated as a reproductive sex pheromone produced and secreted by the male (Li et al., 2002, Siefkes et al., 2003) (See section 1.8 discusion).

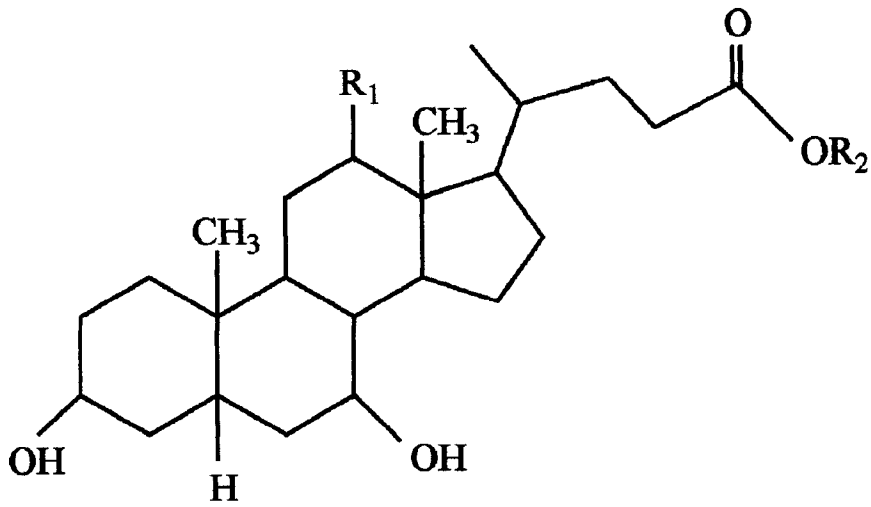

Figure 6. Bile acids are C_{24} compounds. Various substituents at the R_1 and R_2 positions on the compound yield unique bile acids.

1.6.4 Fish Pheromones: Steroids

Androgen steroids are C_{19} compounds and oestrogen steroids are C_{18} compounds. Progestogens are C_{21} steroids (Kime, 1993). Numbering of the steroid nucleus is required in order for precise nomenclature (Figure 7). Substituents lying below the plane of the ring are referred to as α, whereas those that lie above the plane are referred to as β.

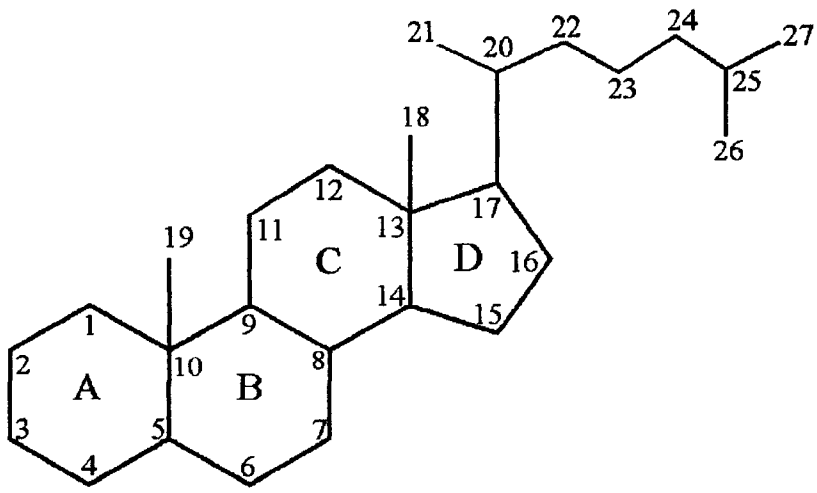

Figure 7. The steroid skeleton indicating the numbering of the rings.

To determine the reproductive steroids that are produced in fish, typically suitable radiolabelled precursors are implemented. This technique is implemented in Chapters 3 and 4 of this thesis, with the *in vivo* incubation of gonadal tissue from the round goby (using [^{3}H]-17-hydroxyprogesterone and [^{3}H]-androstenedione precursors). Tritiated precursors such as [^{3}H]-progesterone, [^{3}H]-17-hydroxyprogesterone, [^{3}H]-androstenedione are incubated with minced tissue and the identity of the products formed is determined by chromatography and microchemical reactions (Kime, 1993). The minced tissue are artificial systems and may not reflect the *in-vivo* situation, where products are continually removed by circulation(Kime, 1993). Although there are limitations, incubation with labeled steroid precursors does give an indication of the enzyme systems present in the tissue and may indicate the presence of novel steroids (Kime, 1993).

Since the studies of Chapters 3 and 4 are focused on the steroid production of putative steroidal reproductive pheromones in the male gonads of the round goby, the discussion here will be limited to reproductive steroids and steroid biosynthesis in the *gonads* of male fish. In male fish, reproductive steroids are typically 5α and 5β reduced. Reduction at the 5th position is very prevelant in perciforms. 5β reduced metabolites predominated when testicular tissue was incubated with either C_{19} or C_{21} precursors of the two periforms, *Rhabdosrgus sarba* (Yeung and Chan, 1985) and *Gobius jozo* (Colombo et al., 1977). 5α reduction predominates in *Glossogobius olivaceus* (Gobiidae) (Asahina et al., 1985) and *Brachydanio rerio* (van den Hurk et al., 1987). Numerous androgen steroids have been identified in fish, but the main ones with known hormonal (i.e. endocrine) roles are testosterone (T) and 11-ketotestosterone (11-KT; Kime, 1993).

The former is found in both male and female fish and is responsible for positive feedback control of pituitary gonadatropin synthesis during gonadogenesis (Crim & Evans, 1983). The latter is only normally found in males and is responsible for stimulating spermatogenesis and male secondary characteristics (Miura *et al.*, 1991).

The addition of a sulfate or glucuronide group at the third postion forms a steroid conjugate. Studies have indicated that steroid conjugates produced in the gonads of the male fish possess pheromonal properties (Colombo et al., 1979; Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988). Both 5β-reduction and the conjugation of steroids are usually mechansims to abolish hormonal activity (Colombo et al., 1980; Kime, 1993). Typically, in mammals, both 5β-reduction and the conjugation of steroids are typically hepatic in location and usually lead to excretion and deactivation of the steroid hormone (Colombo et al., 1980; Kime, 1993). In the larval sea lamprey, a bile acid, petromyzonal sulfate was shown to be produced in the liver (Polkinghorne et al., 2001). Fish are unique in that enzymes for conjugation can be very active within the gonad itself (Kime, 1993). Etiocholanolone glucuronide, produced within the testis of the black goby (*Gobius jozo=G.niger*) has been shown to be an attractant to females (Colombo et al., 1980). Since steroids that are conjugated are more water soluble than the free steroids, conjugated steroids are potentially more likely to be utilized as pheromones in the aqeuous environment of the fish (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996).

1.7 Pheromonal systems in fish: the goldfish (*Carassius auratus*) model system – the use of prostaglandins and steroids.

The most well studied model of a vertebrate pheromonal system is that of the goldfish (*Carassius auratus*). This model suggests the release of specific protaglandins and steroid pheromones by the female gonads to which the male respond. The female releases specific pheromones before and after ovulation; this initiates spawning activity in males. The following five pheromonal compounds have been shown to be released by the female goldfish to induce physiological and behavioral responses in males: androstenedione (AD), 17,20β-Dihydroxy-4-pregnen-3-one (17,20βP), 17,20β-Dihydroxy-4-pregnen-3-one-sulfate (17,20βP-S), Prostaglandin $F^{2\alpha}$ (PGF2α), 15Keto-Prostaglandin $F^{2\alpha}$ (15KPGF2α). In the afternoon and before ovulation, female pituitary gonadotropins stimulate the release of preovulatory AD by the female into the water. Later a surge of preovulatory 17,20βP is released into the water, this dominates the steroidal mixture. Still in the preovulatory phase, the female proceeds to release 17,20βPS; the presence of 17,20βPS now dominates the steroidal mixture presented to the male. By the end of the night, males have greatly increased sperm levels due to exposure to 17,20βP. Hence, the principle function of 17,20βP is to increase male steroidogenesis and milt production, thereby giving exposed males increased fertility by the time of spawning (Dulka et al., 1987a; Sorensen et al., 1989, 1990; Zheng and Stacey, 1997). The reproductive cycle proceeds and females ovulate and steroid levels drop in the plasma. Circulating PGF2α increases in the female which stimulates female reproductive behaviour. Finally, the two postovulatory pheromones, PGF2α and its principle metabolite, 15KPGF2α are released by the female in urine; these two compounds act together as sex pheromones with strong effects in the male (Sorensen et

al., 1998). Free steroids appear to be released nearly exclusively across the gills in goldfish, and conjugated (glucuronidated and sulfated forms) are released in the urine (Sorensen et al., 2000). The possibility of male goldfish releasing pheromones in which females are responsive to has received little attention and there is no evidence that ovulation is influenced by the presence of males (Stacey et al., 1979b). Therefore, in the goldfish, a complete synchrony of events has been established, whereby the female stimulates male spawning behavior.

1.8 Reproductive Pheromonal systems in fish: the Sea Lamprey – the use of bile acids

In the mature male sea lamprey (*Petromyzon marinus*), a bile acid sex pheromone, 7α, 12α,24-trihydroxy-5α-cholan-3-one-24-sulfate is excreted from the gills which acts as a potent sex pheromone for females (Li et al., 2002; Siefkes et al., 2003). Since this bile acid is present in the liver of spermiating males, this is where it is likely synthesized (Li et al., 2002; Siefkes et al., 2003). The exit of this sulfated bile acid to the aqueous environment may represent an evolutionary adaptation and the transport of this compound from the liver to the gills seems to be very efficient. Hepatic veins carry blood directly to the heart and all blood from the heart passes immediately through the gills. The gills possess specialized glandular cells to facilitate placement of the pheromone into the environment (Siefkes et al., 2003).

1.9 Reproductive Pheromonal systems in fish: developments in the Gobiidae – the use of sex steroids

Other evidence for the use of reproductive pheromones is present in species from the subfamily Gobiidae. In Chapters 3 and 4, the objective is to elucidate the chemical structure of a reproductive pheromone(s) that is released by the male to attract females to

its nest during mating. This could offer a means of species management of this invasive species through interfering with its pheromone communication system. Evidence from species of the subfamily Gobiidae indicates that males release reproductive pheromones that attract females.

In the male black goby (*Gobius jozo)*, it has been shown that the male releases a pheromone that induces reproductive behaviour in the female (Colombo et al., 1979, 1980, 1982). The testes of the black goby has been shown to contain a cluster of Leydig cells (i.e. steroid secreting cells), which forms the mesorchial gland. The mesorchial gland is concentrated in the region where the testis is suspended from the body wall by lengthwise mesenteries known as mesorchia (Colombo et al., 1974, 1977,1982). Furthermore, the mesorchial gland was capable of transforming radioactive pregnenolone into conjugated and 5β-reduced steroids (Colombo et al., 1970, 1977) (see figure 4 for structure). One of the steroids shown to be synthesized in this mesorchial gland, etiocholanolone glucuronide, was shown to act as an attractant to gravid females (Colombo et al., 1980). The work of Murphy et al. (2001) indicated that the olfactory system of the round goby is particularly responsive to 5β reduced androgens. Therefore, based on the work of Colombo et al. (1970,1977) and Murphy et al. (2001), the search for reproductive pheromones in the round goby (as carried out in Chapters 2 and 3), was focused towards the identification of 5β reduced C_{19} androgens that were produced in the gonads of the male.

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CHAPTER 2: Olfactory epithelial localization of G_{olf} negative olfactory sensory neurons projecting to medial olfactory bulb glomeruli in the larval sea lamprey (*Petromyzon marinus L.*)

2.1 Introduction

The sea lamprey, *Petromyzon marinus*, is a jawless vertebrate fish belonging to the class Agnatha. It is monorhinic with the single nostril located on the dorsal surface of the head. The nose of the sea lamprey contains the olfactory epithelium with olfactory sensory neurons (OSNs) on the posterior surface of the ventral region of the nasal cavity. The OSNs are bipolar, possessing axons that extend caudally and converge into the olfactory nerve. The olfactory nerve projects into glomerular units of the olfactory bulb, where OSN axon terminals form synapses with dendrites of mitral cells.

The olfactory glomeruli are believed to function as basic modules in information processing and odourant detection (Shepherd, 1994), but a full understanding of the principles of glomerular organization is lacking (Hildebrand and Shepherd, 1997; Friedrich and Korsching, 1998; Xu et al., 2000). Six organizational glomerular territories exist in the larval sea lamprey: dorsal cluster (40-100 μm depth), dorsal ring (100-200 μm depth), anterior plexus (225-475 μm depth), lateral chain (225-475 μm depth), medial (350-575 μm depth), ventral ring (500-675 μm depth), and ventral cluster (700-775 μm depth) (Frontini et al., 2003). All glomerular territories except the medial glomeruli are G_{olf}-IR (Frontini et al., 2003). This suggests a spatial organization of glomerular units that is dictated by functional parameters. The olfactory GTP binding protein, G_{olf}, is cAMP dependent and linked to olfactory receptors (Jones and Reed, 1989); and has been shown to be fundamental in olfactory sensory trandsduction and olfactory responses (Belluscio et al., 1998). The OSN projections into these discrete medial glomeruli may

be spatially segregated in the olfactory epithelial sheet of the peripheral olfactory organ. Information is lacking with respect to the spatial organization of olfactory information within subsets of glomeruli and within the peripheral olfactory organ. In the present study, the OSN projections into the medial, G_{olf}-non-IR glomeruli are examined. This will further our understanding of the spatial organization of olfactory information between the olfactory bulb and the peripheral olfactory organ.

2.1.2 Odourant signal transduction and the presence of G-protein coupled receptors in OSNs

The G-protein, G_{olf} is essential for olfactory responses in mammals (Belluscio et al., 1998). The G_{olf} protein is expressed in many vertebrate OSNs such as the amphibian *Xenopus laevis* (Mezler et al., 2001) and teleost fish (Hansen et al., 2003). In vertebrate olfactory systems, alternate G proteins are expressed in different sub-populations of OSNs that extend axons into spatially distinct glomerular units (Jia and Halpern, 1996; Hansen et al., 2003). This would indicate the presence of different sub-populations of OSNs in the olfactory epithelium that implement different signal transduction mechanisms for the detection of different odourant molecules. In the catfish *(Ictalurus punctatus)*, the projections of ciliated G_{olf}-IR OSNs are primarily to medial (bile salt receptive) and ventral (amino acid receptive) regions of the olfactory bulb (Hansen et al., 2003). Furthermore, in this catfish, microvillous OSNs that express $G_{\alpha o}$ and $G_{\alpha q/11}$ project to the dorsal region of the olfactory bulb where responses to amino acids and nucleotides predominate (Hansen et al., 2003).

In the mammalian vomeronasal organ, receptor neurons that express the G proteins $G_{i\alpha 2}$ and $G_{o\alpha}$ project to segregated glomeruli within the accesory olfactory bulb

(Jia and Halpern, 1996). In mammals, the accessory olfactory bulb is located on the dorso-posterior surface of the main olfactory bulb (Jia and Halpern, 1996). Also, in mammals, a subset of OSNs that selectively express elements of the cyclic guanosine monophosphate (cGMP) signal transduction pathway project to a distinct group of glomeruli in the olfactory bulb, the "necklace glomeruli", which resemble a beaded necklace encircling the caudal region of the olfactory bulb (Juilfs et al., 1997). This indicates that cGMP in these OSNs that project to the necklace glomeruli may have an important function in olfactory signaling. These "necklace glomeruli" are unique since they have been associated with pheromone induced responses in rat pups (Teicher et al., 1980; Greer et al., 1982). In the lamprey (*Lampetra fluviatilis*), the immunocytochemical distribution of calretinin, a calcium-binding protein, was localized within sub-populations of OSNs in the olfactory epithelium and within some olfactory bulb glomeruli (Pombal et al., 2002). Four groups of glomeruli were identified in the olfactory bulb of the lamprey (*Lampetra fluviatilis*), whereby variable levels of calretinin-IR are displayed, ranging from strong IR to no IR (Pombal et al., 2002). Likewise, in the larval sea lamprey, there exists a unique pattern of expression of G_{olf} throughout the olfactory system, in that G_{olf} is not being expressed in the medial glomeruli (Frontini et al., 2003). This indicates a possible subpopulation of neural innervation from the olfactory epithelium to the medial glomeruli that does not use G_{olf} in signal transduction. Since G_{olf} is absent in medial glomeruli of the olfactory bulb, this suggests a putative independent pathway for odourant coding in the olfactory bulb. The medial olfactory bulb glomeruli and the medial olfactory tract has been associated with pheromone perception in teleosts (Stacey and Kyle, 1983; Kyle et al., 1987; Sorensen et al., 1991; Friedrich and Korsching, 1998;

Weltzien et al., 2003). Therefore, the medial glomeruli and the corresponding OSNs of the OE may represent an independent pathway for detecting pheromonal compounds in lampreys.

2.1.3 The organization of the olfactory system: topographic projections between the olfactory epithelium and the olfactory bulb

Rhinotopy occurs when OSNs in a particular region of the epithelium innervate a distinct part of the olfactory bulb. In the hamster, fluorescent retrograde tracing (implementing fluorescent stilbene isothiocyanates) studies have indicated the presence of rhinotopy in the mammalian olfactory system (Schoenfeld et al., 1994). In the mammalian olfactory system, OSNs in the ventral-medial olfactory epithelium project to the glomeruli in the ventral-medial main olfactory bulb, OSNs in the dorsal-medial olfactory epithelium projects to the dorsal-medial main olfactory bulb, dorsal-lateral to dorsal-lateral main olfactory bulb, etc. (Schoenfeld et al., 1994). Rhinotopy describes this zonal mapping of OSN projections to the olfactory glomeruli in the olfactory bulb. Such topographic projections from the nose to the bulb may be fundamental in forming a sensory space and a spatial code that organizes odourant quality in the olfactory system (Schoenfeld et al., 1994).

The connectional topography between the olfactory epithelium and the olfactory glomeruli also has been studied in the zebrafish (*Danio rerio*) olfactory system through implementing retrograde tract tracers (1,1'-dioctadecyl-3,3,3',3'-tetrameylindocarbocyanine perchlorate, DiI) (Baier et al., 1994). In the adult zebrafish, the glomerular layer consists of 80 glomeruli, categorized into 18 glomerular groups, that are invariant from animal to animal (Baier and Korsching, 1994). OSN projections to the olfactory bulb are random in the zebrafish; the existence of distinct zones of the

epithelium projecting to distinct zones of the olfactory bulb glomeruli was not apparent. Specifically, in the zebrafish, it was found that OSNs projecting into the ventroposterior glomerulus were widely dispersed over the olfactory epithelium (Baier et al., 1994). These researchers chose to use the ventroposterior glomerulus for practical reasons. Relative to the other glomerular units, it is large (Baier and Korsching, 1994: Table 1). Also, it is easily accessible for microinjection since it is isolated from the rest of the glomeruli in the ventral-posterior aspect of the olfactory bulb which restricts the dye label to this particular glomerulus (Baier et al., 1994). These findings in the zebrafish are consistent with findings in the rainbow trout (*Oncorhynchus mykiss*). In the rainbow trout, anterograde tracing studies indicated that regardless of where horseradish peroxidase was applied in the peripheral olfactory organ (i.e. olfactory epithelium), labeled axons were never restricted to a subregion of the glomerular layer (Riddle and Oakley, 1991). Furthermore, retrograde tracing studies indicated that regardless of which glomerular sites were injected (Lumafluor injections included anterior medial, anterior lateral, lateral or posterior lateral region of the dorsal half of the olfactory bulb) labeled OSNs were widely dispersed in the olfactory epithelium (Riddle and Oakley, 1991). These studies indicate that in both the rainbow trout and the zebrafish, projections from the olfactory epithelium to the olfactory bulb are non-topographically ordered, and the organization of odourant information seems to begin in the glomerular units of the olfactory bulb. In zebrafish, the medial glomeruli respond to stimulation by bile acids; the anterior and lateral glomeruli respond to amino acids; and the medial ventral glomeruli respond to a reproductive prostaglandin pheromone (Friedrich and Korsching, 1997). Furthermore, in rainbow trout, the lateroposterior region of the olfactory bulb is

specifically responsive to amino acids and the medial area of the olfactory bulb is specifically responsive to bile acids (Hara and Zhang, 1996). Therefore, these data in the zebrafish and the rainbow trout indicate that OSNs with common odourant receptors are dispersed within the olfactory epithelium converge on common glomeruli located in segregated regions of the olfactory bulb. This contrasts to the topographic ordering observed in mammals such as the rodent (Schoenfeld et al., 1994). Some early evidence in the carp (*Cyprinus carpio*) indicated a spatial segregation of OSNs from specific areas of the olfactory epithelium projecting to particular regions of the olfactory bulb (Sheldon, 1912). Specifically, the medial bundle of the olfactory nerve is derived from the rostral lamellae and the lateral bundle is derived from the caudal lamellae (Satou et al., 1983). Besides this early study conducted in the carp, studies in fish indicate an olfactory model whereby topographic projections between the olfactory bulb and olfactory epithelium is lacking (Hara and Zhang, 1996). It is the objective of this study to determine whether the larval sea lamprey, an organism shown to differentially express G_{olf} in its olfactory system (Frontini et al., 2003), possesses diffuse projections like the zebrafish and rainbow trout, or topographically ordered projections like the rodent.

Studies from conventional neuroanatomical tract tracing studies in mammals have established a zonal map between the epithelium and the olfactory bulb (i.e. Schoenfeld et al., 1994). The idea of a zonal map in the olfactory system has been extended through determining the expression of odourant receptor genes and cell surface molecules which are concentrated on olfactory axons (Mori et al., 1999; Yoshihara et al., 1997). The presence of sharply bounded zones was demonstrated using monoclonal antibodies reactive to specific cell surface proteins, which differentially label the dorsomedial versus

the ventrolateral olfactory epithelium and the axonal projection onto the bulb (Mori et al., 1999; Yoshihara et al., 1997; Schwob and Gottlieb, 1988). In the mouse it has been indicated the neural cell adhesion molecule, OCAM, is strongly expressed in the ventral olfactory epithelium which projects to the ventral and lateral glomeruli in the olfactory bulb. Furthermore, the dorsolateral olfactory epithelium, which does not express OCAM, projects to the dorsomedial olfactory bulb (Christensen et al., 2001). In mammals, the OE is divided into four zones; each zone contains OSNs that express specific olfactory receptor genes (Ressler et al., 1993; Vassar et al., 1993). Furthermore, this model of zonal gene expression suggests that OSNs that express a certain olfactory receptor converge onto distinct glomeruli (Mombaerts, 1999). This idea is consistent to the zonal mapping depicted in the hamster, implementing neuronal tract tracers, whereby OSNs in specific areas of the olfactory epithelium project to distinct glomerular units in the olfactory bulb (Schoenfeld et al., 1994).

Taken together, many studies have indicated that the olfactory system is organized on the basis of spatial division of OSN populations that project to the distinct olfactory glomeruli. OSNs in the olfactory epithelium segregate on the basis of odourant receptor type and olfactory bulb glomeruli may receive inputs from OSNs that respond to similar odourants. Physiological data also support the spatial projection of OSNs onto specific olfactory glomeruli; for example, in zebrafish, the medial and lateral glomeruli are responsive to different odourant molecules (Friedrich and Korsching, 1997).

2.1.4 The organization of the olfactory system: the use of anatomical space for chemoreception

The vomeronasal organ (VNO) is a chemoreceptive structure located at the base of the nasal septum of most terrestrial vertebrates (Halpern, 1987). The vomeronasal system consists of a peripheral organ, with epithelium that contains its own sensory neurons that form the vomeronasal nerve that projects to glomeruli in the accessory olfactory bulb (AOB) (Halpern, 1987). The epithelium of the VNO is separate from the olfactory epithelium, which also contains sensory neurons that project to the main olfactory bulb (MOB). It is this spatially separated vomeronasal system that has been implicated in processing pheromonal information in many mammalian species (Halpern, 1987). Unlike vertebrates, fish do not have a VNO; however, there is the possibility that the vomeronasal system is present in some fishes in a form that has not yet been recognized (Eisthen, 1992). The functional division of the olfactory system in teleosts may indicate the presence of an analogous system, similar to the VNO identified in many mammals (Eisthen, 1992). The spatial division of the olfactory system into medial and lateral functional elements in teleost fish may be similar to the functional division observed between the olfactory and vomeronasal systems (Eisthen, 1992). In the goldfish olfactory system, the medial olfactory tract responds to reproductive sex pheromones and may be considered homologous to the tetrapod VNO; furthermore, the lateral olfactory tract may be considered homologous to the tetrapod main olfactory system (Dulka, 1993). The medial olfactory tract is responsible for reproductive behaviour in male goldfish (Stacey and Kyle, 1983; Kyle et al., 1987). Furthermore, the medial olfactory tract of the male goldfish selectively elicits electrical activity in responce to sex pheromones

(Sorensen et al., 1991). As well, goldfish were also found to be unable to distinguish between different amino acids after cutting the lateral olfactory tract (von Rekowski and Zippel, 1993). The olfactory system of other teleost fish has displayed an anologous functional and spatial division (Friedrich and Korsching, 1997; Hara and Zhang, 1996; Laberge and Hara, 2003; Weltzien et al., 2003). In the zebrafish, a very different response pattern in the medial and lateral olfactory bulb glomeruli has been observed. The medial glomeruli respond to stimulation by bile acids; the anterior and lateral glomeruli respond to amino acids; and the medial ventral glomeruli respond to a reproductive prostaglandin pheromone (Friedrich and Korsching, 1997). Likewise, in the Atlantic salmon (*Salmo salar)*and the rainbow trout (*Ocorhynchus mykiss*), it has been shown that amino acids selectively stimulate the lateroposterior olfactory bulb and bile acids selectively stimulate the medial olfactory bulb (Hara and Zhang, 1996). In the lake whitefish (*Coregonus clupeaformis*), putative reproductive pheromones have been shown to selectively stimulate a transition area in the olfactory bulb-telencephelon transition area (Laberge and Hara, 2003). When the medial olfactory tract of the crucian carp (*Carassius carassius*) is cut in males, they exhibited reduced reproductive behaviour (Weltzien et al., 2003). This suggests that the medial olfactory tract of the crucian carp mediates reproductive behaviour. It has also been shown that the lateral olfactory tract mediates feeding responses in the crucian carp (Hamdani et al., 2001).

Taken together, odourant coding for putative pheromones in fish is processed through independent, spatially segregated pathways in the olfactory system. It is possible that the medial olfactory system in teleosts is functionally homologous to the vomeronasal system of higher vertebrates. Whether the medial olfactory bulb glomeruli

in the sea lamprey, which lack G_{olf}-IR, represents such a unique functional subset in the olfactory system is yet to be determined.

In this study, we investigated the olfactory epithelial distribution of OSNs projecting to the medial glomeruli, that do not express G_{olf}. Micro-injections of fluorescent dextran (3000MW) into this distinct area of the olfactory bulb was completed. Since the medial glomeruli do not express G_{olf}, their corresponding OSNs in the olfactory epithelium is not expected to express G_{olf}. Dextran labeling was colocalized with respect to the G-protein, G_{olf}, in the olfactory glomeruli and the OSNs in the peripheral olfactory organ. The jawless sea lamprey represents a divergent line of evolution and the organization of its olfactory system may be very different from jawed teleosts. Hence, we predict that the medial, non-G_{olf} expressing glomeruli projects from discrete areas of the olfactory epithelium. This expectation would be consistent to what is known in other olfactory systems, whereby alternate G proteins are expressed in different sub-populations of OSNs that extend axons into spatially distinct glomerular units (Hansen et al., 2003; Jia and Halpern, 1996).

2.2 Materials and Methods

2.2.1 Experimental animals

Year III class larval sea lamprey, *Petromyzon marinus*, were used in this experiment. These specimens were collected by staff of the Sea Lamprey Control Centre (Department of Fisheries and Oceans), Sault Ste Marie, Canada, from four Lake Huron tributaries (Brown's Creek and Root, Garden, and Mindemoya rivers) using an electroshocker. The animals were maintained in flow-through dechlorinated water at 11-

15 °C in the Department of Biological Sciences at the University of Windsor. Forty animals (n=40) were used in this study. The weight range of these animals was 1.13g-5.60g and the length range was 9.20 cm-17.7 cm.

2.2.2 Retrograde labeling of OSNs

The larvae were anesthetized with MS222 (0.05%, Argent Chemical Laboratories, Inc.) and the primary olfactory pathway (olfactory epithelium, olfactory nerve, and olfactory bulb) was exposed via dissection in lamprey Ringer's solution (130mM NaCl, 3mM KCl, 2mM $MgCl_2$, 10mM HEPES, 2mM $CaCl_2$, 5mM glucose), and placed in a perfusion chamber mounted onto the stage of a microscope (Zeiss Axioskop 2FS). The rostral aspect of the preparation was pinned into Sylgard with a needle (Figure 8). The melanocytes and dura were carefully removed with needle forceps. Before the injection process, approximately 100 µl of a 0.05% Triton-X solution in lamprey Ringer's solution was applied to the olfactory bulbs in order to facilitate dye loading in the olfactory bulb glomeruli. The exposure of the olfactory bulbs to the Triton-X treatement lasted approximately 60 seconds. This was followed by thoroughly rinsing the tissue preparation with lamprey Ringer's solution that lacked Triton-X.

Dextran solution (1-mg/mL dextran in 0.1M $NaHCO_3$, fluorescein, 3000MW anionic, lysine fixable, D3306, Molecular Probes) was injected into specific olfactory bulb territories with a glass micropipette under pressure (10psi, 9ms pulse) applied by a Picospritzer II (Parker Hannifin Corp.). The glass micropipettes were prepared with a vertical pipette puller (KOPF™ model 720; $3_{1/2}$" Drummond capillaries #3-000-203-G/X). Tips were broken-off to a diameter of approximately 50 µm under a dissecting

microscope and filled with the dextran solution through capillary action. The dye was injected into medial glomeruli (G_{olf}-non-IR), located at depth range of 350 μm to 550 μm (Frontini et al., 2003). Non-medial (G_{olf}-IR) glomeruli (which included the dorsal cluster, dorsal ring, anterior plexus, lateral chain and ventral ring) in control animals were also injected. Our hypothesis is that medial glomeruli will back-fill a spatially distinct subset of OSNs in the olfactory epithelium. As a means of comparison, non-medial glomeruli were also injected to determine the location of back-filled OSNs in the olfactory epithelium. The OSN projections into medial and non-medial glomeruli are expected to be spatially segregated in the olfactory epithelium. During the microinjection process, the medial area of the olfactory bulb was punctured with the pulled glass micro-pipette and injected with the dextran. Non-medial glomerular injections were completed in a similar fashion (Figure 8).

To allow retrograde movement of the dye into the olfactory epithelium, the injected olfactory pathway preparation was incubated in lamprey Ringer's solution for 24 hours at 4 °C in the fridge in 1 inch diameter petri dish wells. The tissue was then fixed in 4% paraformaldehyde (see appendix) solution for 24 hours, cryoprotected in a sucrose gradient solution (10-20-30% in phosphate buffer, see appendix) and sectioned with a cryostat (Microm, Heidelberg), with sections 20 μm thick. The position of the dextran labeling in the olfactory bulb glomeruli was confirmed by fluorescence microscopy. Of the 40 animals used in this study, 9 of the animals had successful back-filling of OSNs. In many of the injections, they were considered unsuccessful since either the tissue became necrotic, inappropriate regions of the olfactory bulb were injected (i.e. outside glomerular territories) or back filling of OSNs did not occur (see appendix for mass,

length and sex of these injected preparations). Five preparations (M1-M5) were successfully injected into medial glomeruli. Four preparations (NM1-NM4) were injected into the non-medial glomeruli (which included the dorsal cluster, dorsal ring, anterior plexus, lateral chain and ventral ring) (Table 1).

Table 1. Details of the specimens used in this study. "Preparation number" indicates the code given for each specimen, given on the basis of the specimen having either a medial (M) or non-medial (NM) glomerular injection. "Animal code" refers to an internal coding system used for tracking the length and weight of each larval sea lamprey used in any study (including this one) within the Zielinski Laboratory. The olfactory bulb injected was indicated for each animal in this study.

Preparation Number	Animal Code	Sex	Weight (g)	Length (cm)	Olfactory Bulb Injected (Right/Left)
M1	L6003	Female	2.42	12.1	Right
M2	L7003	Female	1.54	11.0	Left
M3	L6403	Male	1.38	10.1	Right
M4	L6503	Male	1.85	11.4	Right and Left
M5	L6303	Female	1.73	10.8	Right and Left
NM1	L5903	Female	1.52	10.8	Left
NM2	L6703	Female	2.07	11.5	Right and Left
NM3	L7103	Male	1.58	10.9	Right
NM4	L6603	Female	1.85	10.5	Left

2.2.3 Analysis of tissue sections

After the injected olfactory bulbs and the olfactory epithelium were sectioned on the cryostat, tissue sections were analyzed using a Biorad 1024 laser scanning confocal microscope, with an Argon/Krypton laser. The olfactory bulb glomeruli injected with

dextran were identified and the corresponding labeling of OSNs was localized within the olfactory epithelium.

2.2.4 Analysis of tissue sections.
Olfactory Bulb Analysis - morphometric analysis of the dextran injection.

Morphometry was used to determine the dextran labeling of the olfactory bulb. Every tissue section was analyzed and the dextran labeling data was compiled onto templates of the olfactory bulb. The templates of the olfactory bulb consisted of images of horizontal sections through the olfactory bulbs at an interval of every 60 μm depth (Figure 9). The location of the dextran labeling was compiled onto each representative section on the template (Figure 9). The glomeruli were categorized into 7 territories (Figure 10), according to Frontini, et al. (2003), using diagrams of the olfactory bulb depicting glomerular territories. The shape of the injection was constructed by transposing information garnered from the olfactory bulb templates (Figure 9) (see results, Figures 12-20).

2.2.5 Analysis of tissue sections.
Olfactory bulb analysis - the co-localization of the dextran injection with respect to olfactory bulb glomeruli using GS-1 isolectin B_4 histochemistry.

After the micro-injection of dextran into the olfactory bulb, and after the fixation and sectioning processes, GS-1 isolectin B_4 histochemistry was implemented with selected preparations (Tobet et al., 1996). GS-1 isolectin B_4 histochemistry labels all of the olfactory bulb glomeruli in the larval sea lamprey (Frontini et al., 2003). This labeling technique co-localized the olfactory bulb glomeruli with the dextran injection site; hence, this double label technique highlighted which glomeruli were labeled. The sections were washed in 0.1 M PBS (see appendix) with three ten minute intervals in

between each step. The slides were treated with avidin/biotin blockers for 15 minutes to eliminate any lectin binding to endogenous avidin. GS-1 isolectin B_4 biotin-conjugated (Griffonia simplicifolia lectin I-isolectin B_4, Vector, Burlingame, CA, B1205; 10ug/mL in 0.1M PBS, pH 7.5) was applied onto the sections overnight at 4°C. After rinsing, the slides were incubated with Texas Red avidin-fluorescein DCS (1:100, Vector, A2016) for 1 hour, washed in PBS three times in the dark, and mounted with Vectashield ™ (Vector, Burlingame, CA).

2.2.6 Analysis of tissue sections.

Olfactory bulb, olfactory epithelium analysis – co-localization of dextran labeled glomeruli and OSNs with respect to G_{olf} immunoreactivity.

After the micro-injection of dextran into the olfactory bulb, and after the fixation and sectioning processes, G_{olf} immunocytochemistry was performed on all injected preparations in order to determine if the dextran injection site contained G_{olf}-IR OSNs in the olfactory bulb glomeruli and the olfactory epithelium. We expected to find the G_{olf}-non-IR OSNs in the olfactory epithelium in preparations with back-filled medial glomeruli (G_{olf}-non-IR). Slides with tissue sections were incubated in diluted normal goat serum for 20 minutes, then in primary antiserum raised in rabbits against G_{olf} (1:500; Santa Cruz Biotechnology, Santa Cruz, CA) in 0.1M PBS, pH 7.4, containing 0.1% Triton X-100 overnight at 4°C. The sections were rinsed in PBS, incubated in Alexa 568 goat antirabbit IgG (Molecular Probes, Eugene, OR; 1:100 in PBS, pH 7.4) for 1 hour and rinsed in PBS.

The analysis of the spatial distribution of dextran back-filled OSNs that were either G_{olf}-IR OSNs or G_{olf}-non-IR in the olfactory epithelium was completed after the application of the G_{olf} immunocytochemistry. The olfactory epithelium was divided into

four regions according to morphological differences at four different depths: region 1 (dorsal: 0-120 μm), region 2 (dorsal: 120-300 μm), region 3 (olfactory nerve: 300-420 μm), and region 4 (ventral: 420-720 μm) (Figure 11). Region 3 and 4 are distinct. The olfactory nerve projects caudally in region 3. The accessory olfactory organ protrudes from the caudal surface of region 4. In each region of the olfactory epithelium, the distribution dextran back-filled OSNs and their G_{olf}-IR (negative IR or positive IR) was determined. This indicated the spatial distribution of G_{olf}-IR and G_{olf} –non-IR OSNs that were back-filled with dextran. Dextran back-filled OSNs in the olfactory epithelium that are G_{olf}-IR should have a green interior (indicating dextran back-filling) and should have red outlining the cell membrane (indicating G_{olf} positive reaction). Dextran back-filled G_{olf}-non-IR OSNs should have a green interior and lack red outline of the cell membrane (indicating a G_{olf} negative reaction) (see results, Figure 21).

2.2.7 Analysis of tissue sections.

A triple label indicating the dextran micro-injection is exclusively into medial, G_{olf}-non-IR glomeruli

With selected animals, a triple label was implemented that was composed of the dextran micro-injection (green, 488nm absorbance), GS1B_4 lectin histochemistry (red, 568nm absorbance), and G_{olf} immunocytochemistry (647nm absorbance). For this triple label, the dextran micro-injection was performed first, followed by GS1 B_4 lectin histochemistry. Lastly, G_{olf} immunocytochemistry was completed by implementing Alexa fluor 647 Cy5 goat antirabbit IgG as explained in section 2.2.6 (Molecular Probes, Eugene, OR; 1:100 in PBS, pH 7.4; 1 hour and rinsed in PBS). Negative controls, with primary antibody omitted from the staining procedure were included with each immunocytochemical preparation. The GS1B_4 lectin histochemistry and G_{olf}

immunocytochemistry labeling was implemented to confirm the micro-injection of G_{olf}-non-IR medial glomeruli. The $GS1B_4$ lectin histochemistry labeled all glomerular units. The G_{olf} immunocytochemistry labeled all glomerular units except for the medial G_{olf}-non-IR glomeruli. Therefore, dextran labeling of the medial glomerular units was confirmed when the medial glomeruli double labeled with dextran and $GS1B_4$ lectin (a G_{olf} negative reaction is displayed). The remainder of the glomeruli (dorsal cluster, dorsal ring, anterior plexus, laterial chain, ventral cluster, ventral ring) were expected to double label with both a GS1 B_4 positive reaction and G_{olf}-IR.

2.2.8 Production of micrographs

The confocal images of the olfactory bulb glomeruli and the OSNs were obtained using a Biorad 1024 laser scanning confocal microscope, with an Argon/Krypton laser. The BioRad PIC format was converted to TIFF format with Confocal Assistant. The photomicroscope images were assembled into figures and labeled with Adobe PhotoShop (Mountain View, CA).

Figure 8. Preparation of the olfactory pathway for the micro-injection procedure. Injection into the medial glomeruli was directed towards the area indicated as "M" in the picture. Injection into non-medial glomeruli was directed towards the area indicted as "NM" in the picture. The olfactory nerves (filled arrowheads) are shown projecting from the monorhinic nostril to the olfactory bulb. Most of the meninges were carefully removed in this picture with needle forceps. Image was obtained at 5X magnifaction. Scale Bar is 0.5mm. The micro-injection process was completed in the field of view shown here, as viewed by a Zeiss Axioscope 2FS.

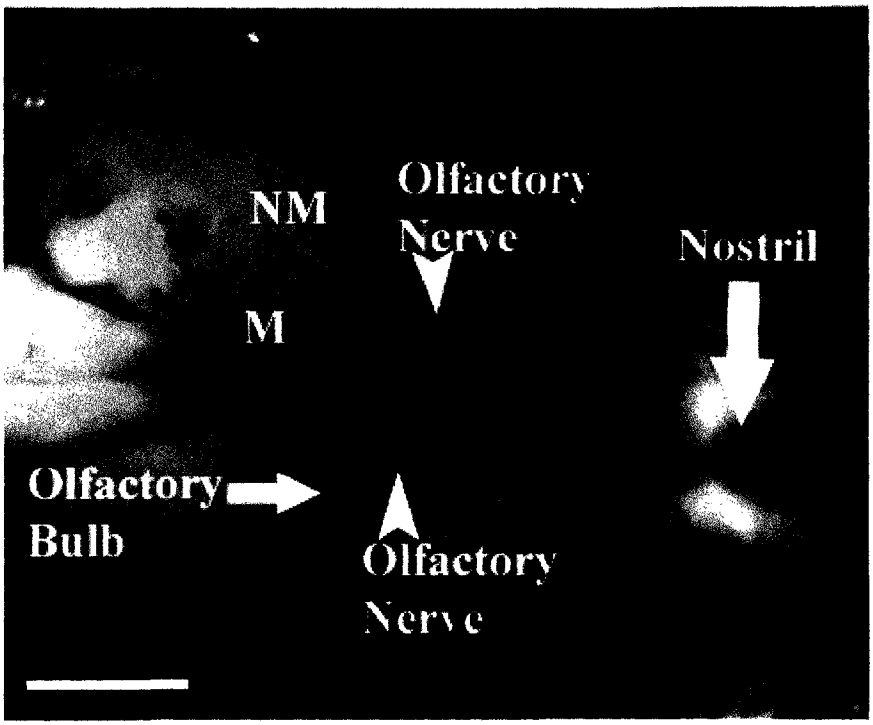


Figure 9. The template of the olfactory bulb used for identifying glomerular territories labeled with dextran. Pictures of serial horizontal sections were taken every 60 μm of the olfactory bulb (OB) and olfactory epithelium (OE). The location of dextran labeling from 3 sections (each 20 μm) was compiled onto each image. Images were obtained at 5X magnifaction, with a Zeiss Axioscope 2FS. Scale bar=0.5mm (at bottom left).

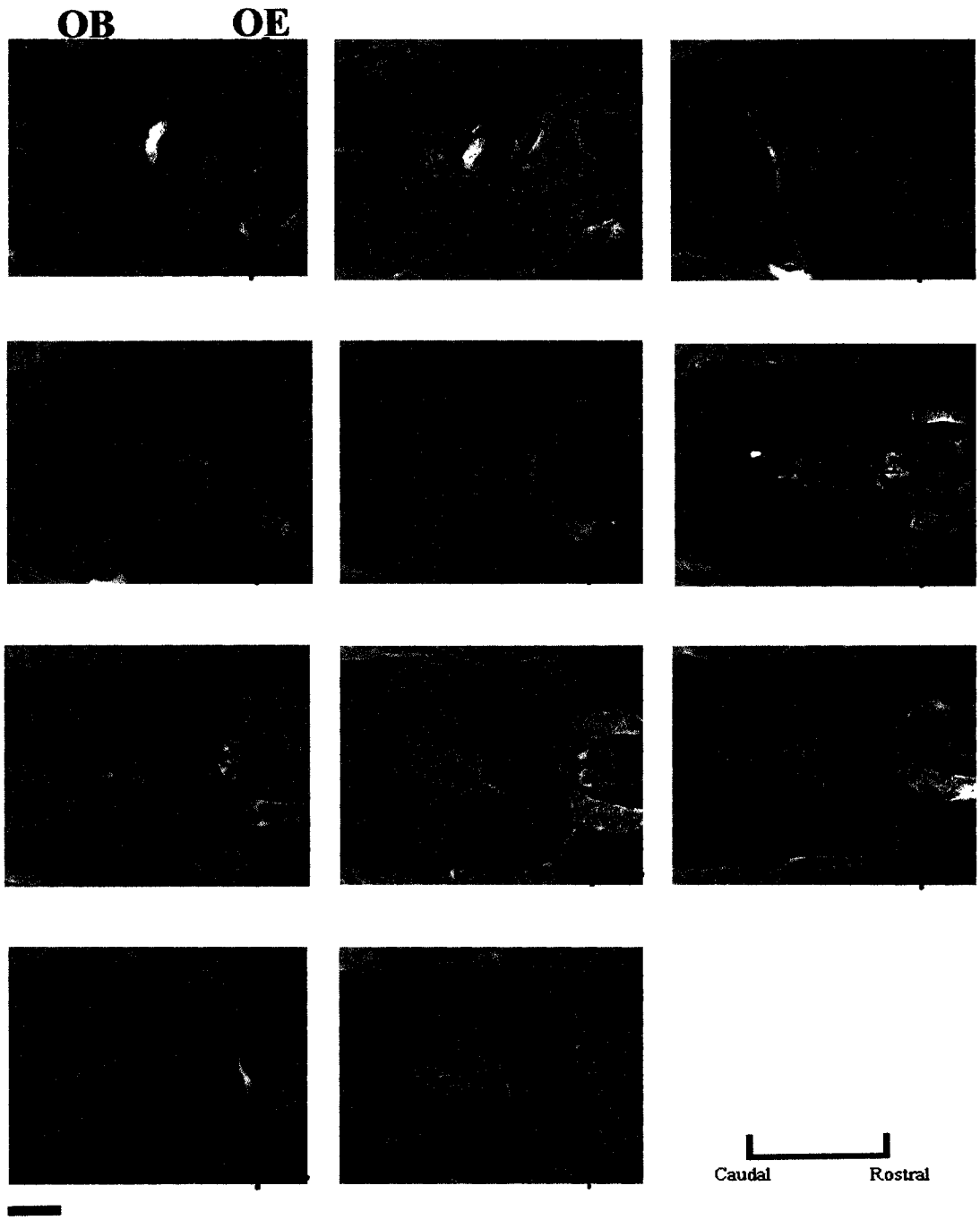


Figure 10. A schematic diagram of a cross-section of the olfactory bulb indicating the depth of the olfactory bulb glomeruli. From the information gained in Figure 1, the shape of the dextran injection was transposed onto this cross-section of the olfactory bulb.

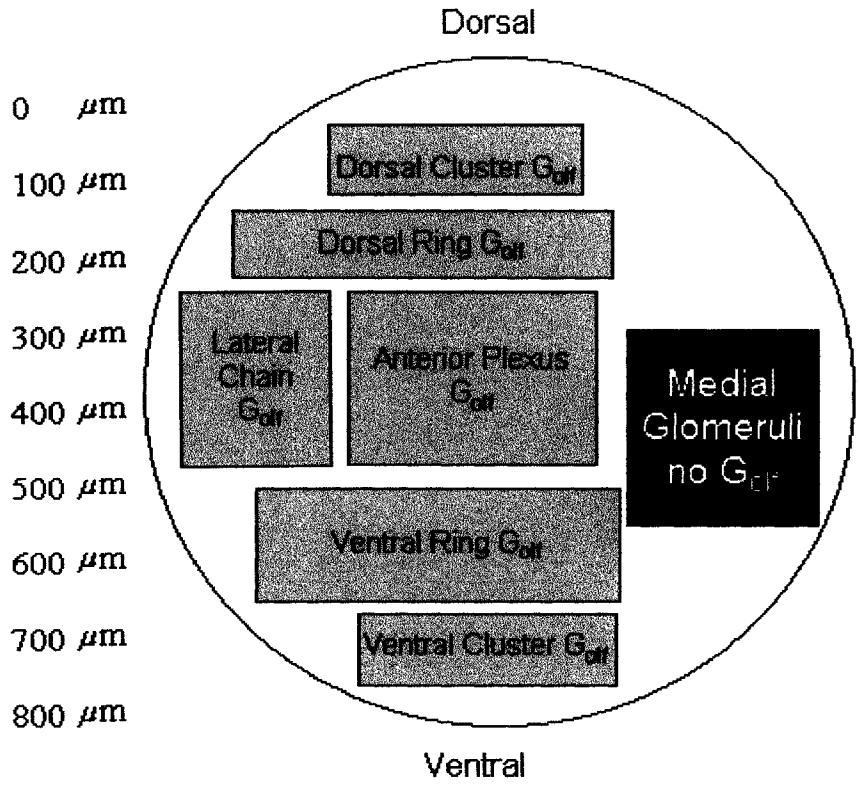
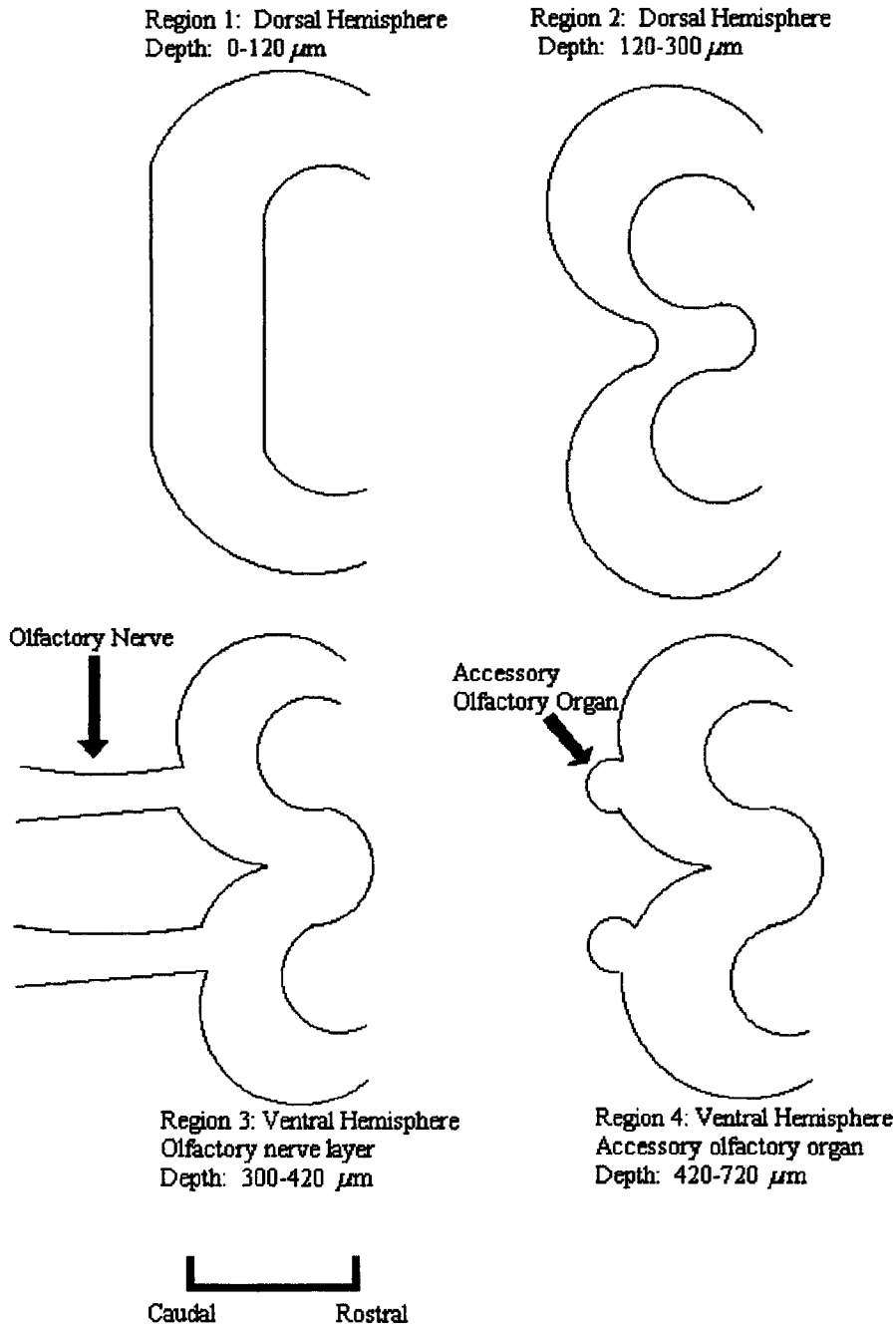


Figure 11. Schematic diagrams of horizontal sections of the olfactory epithelium. Each depth designated has a signature characteristic shape. Each 20 μm section was analyzed and all OSNs labeled with dextran from each animal was compiled onto these diagrams of the olfactory epithelium. The representative olfactory epithelial depth is indicated next to each of the four depths. The dorsal hemisphere includes the most dorsal aspect of the olfactory epithelium until a depth of 300 μm. The ventral hemisphere begins at olfactory nerve layer (300 μm) and includes the most ventral aspect of the olfactory epithelium.



2.3 Results

The olfactory epithelial location of OSNs that project into medial glomeruli and non-medial glomeruli of the olfactory bulb in the larval sea lamprey was established. This was accomplished by injecting specific glomerular territories with fluorescent dextran and back filling OSNs. Histochemical and immunocytomical techniques were implemented to co-localize the dextran micro-injection in the olfactory bulb glomeruli and the OSN axon terminals.

The depth of the each injection in the olfactory bulb was determined and the dextran labeled glomeruli were recorded (Figures 12-20). The position of dextran labeled OSNs in the olfactory epithelium and their respective G_{olf}-IR was mapped onto zonal representations of the olfactory epithelium that was based on depth. From this, the olfactory epithelial distribution of G_{olf}-IR and G_{olf}- non-IR OSNs was determined for each injection (Table 2, Table 3, Figures 12-20).

2.3.1 A subset of G_{olf}-non-IR OSNs in the ventral hemisphere of the olfactory epithelium projects to the medial glomeruli

G_{olf}-non-IR OSNs were spatially segregated in the ventral hemisphere of the peripheral olfactory organ (Table 2; Figures 12-16). *Medial injections* (n=5) displayed G_{olf}-non-IR OSNs exclusively localized in the ventral hemisphere (300-720 μm) of the olfactory epithelium (Table 2, Figures 12-16). G_{olf}-non-IR OSNs were found only between depths of 300-720 μm of the olfactory epithelium (Table 2, Figures 12-16). This

indicates that the medial, G_{olf}- non-IR glomeruli project to a spatially conserved subset of OSNs in the ventral hemisphere of the peripheral olfactory organ.

Preparations M1-M5 all had medial glomerular injections, however, each of these preparations had at least one other glomerular territory injected (Table 2, Figures 12-16). In these preparations, the G_{olf} antibody was applied to the entire primary olfactory pathway, in order to indicate dextran back filled G_{olf}-non-IR OSNs in the olfactory epithelium (Figure 21) which project into dextran labeled medial glomeruli (G_{olf}-non-IR). In each of the medial injection preparations (M1, M2, M3, M4, M5), all clusters of G_{olf}-non-IR OSNs extended from the ventral hemisphere (300-720 μm depth) of the olfactory epithelium. Each medial glomerular injection preparation had a minimum of 3 G_{olf}- non-IR back filled OSNs and a maximum of 12 G_{olf}-non-IR OSNs back filled in the ventral aspect (300-720 μm) of the olfactory epithelium. This disparity of labeling of OSNs upon a medial glomerular injection could be due to a differing quantity and pattern of labeling of the medial glomeruli in the various preparations. As well, the dextran likely did not equally label all OSN axons evenly.

Preparations M2, M3, M4 and M5 (with medial and non-medial glomerular injections) displayed G_{olf}-IR OSNs dispersed throughout the olfactory epithelium; these back filled G_{olf}-IR OSNs were assumed to project exclusively to the non-medial, G_{olf}-IR glomeruli that were dextran labeled. In preparation M1, only the medial glomeruli (G_{olf}-non-IR) and the anterior plexus (G_{olf}-IR) were injected. In preparation M1, G_{olf}-IR OSNs were detected in the 300-720um depth of the OE which are assumed to project to the G_{olf}-IR anterior plexus glomeruli; G_{olf}-non-IR OSNs were detected in the 420-720um depth of the OE, which are assumed to project to the G_{olf}-non-IR medial glomeruli. Hence,

preliminary data in this one preparation indicates that the anterior plexus may also project exclusively to the ventral aspect of the OE.

Preparations M1 through M5 indicate that G_{olf}-non-IR OSNs are present exclusively in 300-720 μm depth of the OE; preparation M1 indicates that the anterior plexus projects from the ventral aspect (300-420 μm) of the OE. Data indicating that the medial glomeruli project from the ventral aspect of the OE was confirmed in five animals (Table 2, Figures 12-16). Data indicating that the anterior plexus projects to the ventral aspect of the OE was present only in preparation M1 (Table 2, Figure 12). The fact that the medial glomeruli and the anterior plexus may both project to the ventral aspect of the olfactory epithelium is consistent with the fact that these two glomerular territories are located within overlapping depths of the olfactory bulb.

In summary, the ventral aspect of the olfactory epithelium contains OSNs that project to the medial glomeruli (n=5); OSNs in this region of the OE may also selectively project to the anterior plexus (n=1). It is highly likely that the ventral aspect of the olfactory epithelium is occupied by G_{olf}-IR OSNs that project to other non-medial glomerular (G_{olf}-IR) territories; the data garnered from NM1 and NM2 indicates labeling of dorsal glomeruli (dorsal cluster and dorsal ring) and subsequent labeling of OSNs in the ventral OE (Figures 17, 18).

2.3.2 Non medial glomeruli project from diffuse regions of the peripheral olfactory organ.

In preparations with non-medial (G_{olf}-IR) glomerular injections, G_{olf}-IR OSNs were found throughout the olfactory epithelium (ranging from the most dorsal surface of the peripheral olfactory organ to the most ventral surface; Table 2, Figure 8A-D). Four preparations (NM1-NM4) were injected into non-medial (G_{olf}-IR) glomeruli; this served

as a comparison to the medial glomerular injections. G_{olf}-non-IR OSNs were not detected in the olfactory epithelium in these four preparations, as expected.

The G_{olf}-IR OSNs were widely dispersed in the OE in preparations M2, M3, M4, and M5 (Table 1). The clustering of G_{olf}-non-IR OSNs into the ventral aspect of the OE following medial glomerular injections is not simply an artifact of the preparation implemented in this study: preparations M2, M3, M4, M5, NM1, NM2 possess G_{olf}-IR OSNs in the dorsal aspect (0-300 μm) of the OE that project to non-medial glomeruli (Tables 2, 3; Figures 13,14,15,16,17,18). Since G_{olf}-IR OSNs in the dorsal hemisphere of the OE were back filled with dextran in this study when non-medial glomeruli were injected, the results that indicate that the medial glomeruli project exclusively from the ventral hemisphere of the OE, is not simply experimental artifact. Therefore, these preparations that display OSN labeling in the dorsal aspect of the OE disproves the possibility that the dorsal primary olfactory pathway may have been damaged in this experimental preparation.

Although preparation NM1 may indicate that the dorsal ring glomeruli project to all depths of the OE, preparation NM2 contradicts this (Table 2); further investigation is required if this is to be resolved. In preparation NM2, both the dorsal cluster and dorsal ring were injected and dextran back-filled OSNs were found only within the 0-120 μm and 420-720 μm depths of the OE. This contradicts preparation NM1, whereby the dorsal cluster was labeled and all depths of the OE contained dextran back-filled OSNs. It should be noted that different glomeruli of the dorsal ring could have been injected in preparations NM1 and NM2 which could have resulted in the differential labeling of the OSNs of the depths of the OE.

All non-medial glomerular injections (except preparation NM1) had more than one glomerular territory injected. Since none of the non-medial glomeruli were biochemically distinct from one another (i.e. such as the medial G_{olf}-non-IR glomeruli that are biochemically distinct from the G_{olf}-IR non-medial glomeruli), establishing a distinct pattern of OSN projection to any individual non-medial glomerular territory was not possible. Taken together the results indicated that G_{olf}-IR glomeruli projected diffusely throughout all depths of the olfactory epithelium.

In summary, G_{olf}-non-IR OSNs, which are segregated in the ventral hemisphere of the peripheral olfactory organ, likely project exclusively to the G_{olf}-non-IR medial glomeruli in the olfactory bulb. Furthermore, this study suggests that G_{olf}-IR OSNs are widely dispersed in the olfactory epithelium and that they innervate onto G_{olf}-IR glomerular units of the olfactory bulb.

Table 2: The distribution of OSNs in the olfactory epithelium following medial glomerular injections. The distribution of dextran labeled OSNs in the olfactory epithelium was determined with respect to G_{olf} immunoreactivity. Dextran labeled glomeruli are indicated as follows: Dorsal Cluster (DC), Dorsal Ring (DR), Lateral Chain (LC), Anterior Plexus (AP), Medial Glomeruli (MG), Ventral Ring (VR) and Ventral Cluster (VC). The distribution of OSNs that are present in the OE were recorded at four equal depths. The number of dextran labeled OSNs that were G_{olf} immunoreactive and G_{olf} non-immunoreactive were recorded in within each depth. Dextran labeled and G_{olf}-immunoreactive OSNs are designated as "$D+/G_{olf}+$"; dextran labeled and G_{olf}-non-immunoreactive OSNs are designated as "$D+/G_{olf}-$".

Dextran Glomeruli Labeled	OLFACTORY EPITHELIUM REGIONS LABELED (depth)							
	0-120 μm		120-300 μm		300-420 μm		420-720 μm	
	$D+/G_{olf+}$	$D+/G_{olf-}$	$D+/G_{olf+}$	$D+/G_{olf-}$	$D+/G_{olf+}$	$D+/G_{olf-}$	$D+/G_{olf+}$	$D+/G_{olf-}$
M1 (L6003) Glomerular territories labeled: AP, MG					2 Cells		8 Cells	4 Cells
M2 (L7003) Glomerular territories labeled: AP, MG, VR	8 Cells		27 Cells		104 Cells		26 Cells	3 Cells
M3 (L6403) Glomerular territories labeled: DC, DR, MG, VR	127 Cells		127 Cells		639 Cells	6 Cells	326 Cells	1 Cell
M4 (L6503) Glomerular territories labeled: DC, DR, AP, MG, VR, VC	42 Cells		41 Cells		70 Cells	4 Cells	5 Cells	7 Cells
M5 (L6303) Glomerular territories labeled: DR, AP, MG, VR, VC	14 Cells		27 Cells		184 Cells	9 Cells	377 Cells	3 Cells
Total No. Cells Labeled in All Preparations	191 Cells		222 Cells		999 Cells	19 Cells	742 Cells	18 Cells

Table 3: The distribution of OSNs in the olfactory epithelium following non-medial glomerular injections. The distribution of dextran labeled OSNs in the olfactory epithelium was determined with respect to G_{olf} immunoreactivity. Dextran labeled glomeruli are indicated as follows: Dorsal Cluster (DC), Dorsal Ring (DR), Lateral Chain (LC), Anterior Plexus (AP), Ventral Ring (VR) and Ventral Cluster (VC). The distribution of OSNs that are present in the OE were recorded at four equal depths. The number of dextran labeled OSNs that were G_{olf} immunoreactive and G_{olf} non-immunoreactive were recorded in within each depth. Dextran labeled and G_{olf}-immunoreactive OSNs are designated as "D+/G_{olf}+"; dextran labeled and G_{olf}-non-immunoreactive OSNs are designated as "D+/G_{olf}-".

Dextran Glomeruli Labeled	OLFACTORY EPITHELIUM REGIONS LABELED (depth)							
	0-120 μm		120-300 μm		300-420 μm		420-720 μm	
	D+/ G_{olf+}	D+/ G_{olf-}	D+/ G_{olf+}	D+/ G_{olf-}	D+/ G_{olf+}	D+/ G_{olf-}	D+/ G_{olf+}	D+/ G_{olf-}
NM1 (L5903) Glomerular territories labelled: DR	1 Cell		7 Cells		39 Cells		56 Cells	
NM2 (L6703): Glomerular territories labeled: DC, DR	3 Cells						8 Cells	
NM3 (L7103) Glomerular territories labeled: DC, DR, AP, VR					2 Cells		11 Cells	
NM4 (L6603) Glomerular territories labeled: DR, AP, LC							33 Cells	
Total No. Cells Labeled in All Preparations	4 Cells		7 Cells		41 Cells		108 Cells	

2.3.3 The identification of G_{olf}-IR and G_{olf}-non-IR OSNs in the olfactory epithelium

Dextran back-filled OSNs in the olfactory epithelium that were G_{olf}-IR had a green interior (indicating dextran back-filling) and were outlined with red (indicating G_{olf}-IR) (Figure 21). Since G_{olf} is a G protein that resides on the intracellular surface of the OSN, G_{olf} labeling is only on the membrane of the OSN. These G_{olf}-IR OSNs in the olfactory epithelium project to non-medial, G_{olf}-IR glomeruli in the olfactory bulb. G_{olf}-non-IR OSNs that were dextran back-filled in the olfactory epithelium had a green interior (indicating dextran back-filling) and lacked a red outline (a G_{olf} negative reaction) (Figure 21). These G_{olf}-non-IR, OSNs localized in the ventral hemisphere of the olfactory epithelium, project to the G_{olf}-non-IR medial glomeruli in the olfactory bulb.

2.3.4 A triple label indicating the dextran micro-injection is exclusively into medial, G_{olf}-non-IR glomeruli

With selected preparations that had dextran injections into the medial, G_{olf}-non-IR glomeruli, G_{olf} immunocytochemistry and $GS1B_4$ lectin histochemistry were implemented to confirm the specific labeling of this medial subset with dextran and the absence of G_{olf}-IR. The medial glomeruli show a G_{olf} negative reaction (G_{olf}-non-IR) and are single labeled with the dextran injection (Figure 22A) when G_{olf} immunocytochemistry is employed. This indicates that the medial glomeruli are labeled with dextran (Figure 22A). The medial glomeruli double labeled with dextran (green) and $GS1B_4$ lectin (red), which stains all glomeruli (Figure 22B). This double label is displayed as a slight yellow hue, which is the result of the colour merge of red and green (Figure 22B). The remaining anterior and lateral glomeruli (that are G_{olf}-

IR) double label with both GS1B$_4$ lectin (red) and G_{olf} (blue). This double label is displayed as purple/pink (Figure 22C).

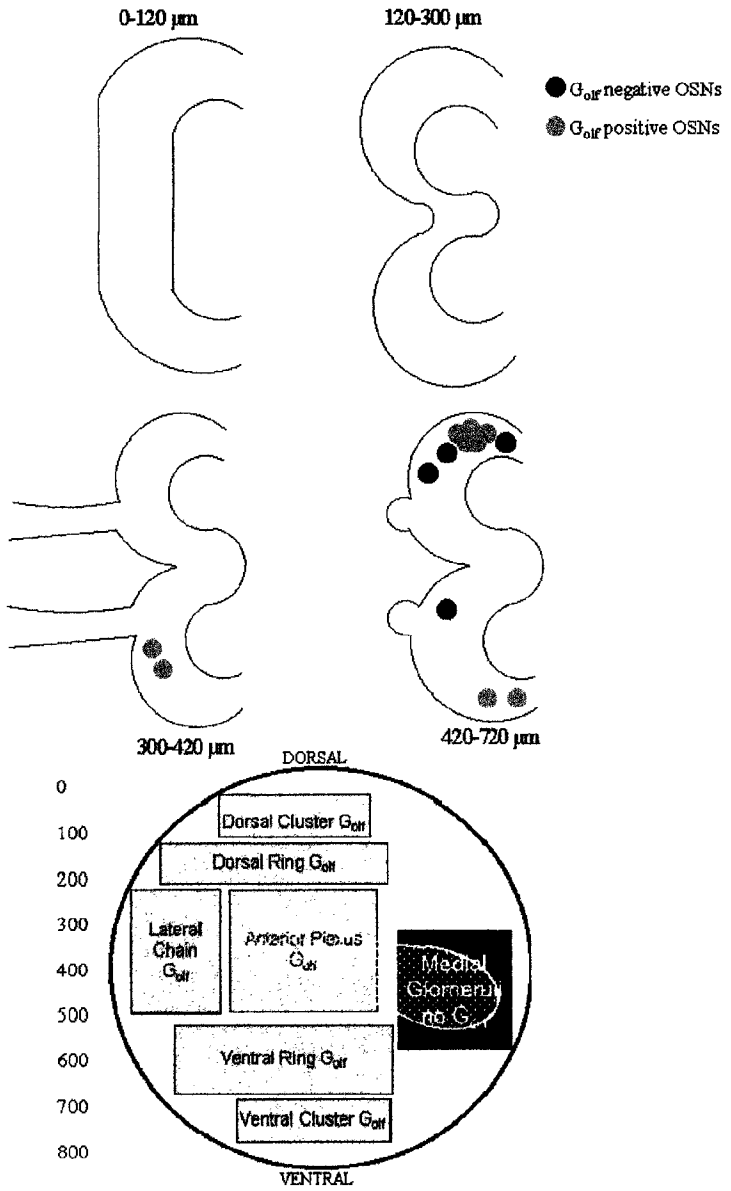


Figure 12. Preparation M1: The ventral hemisphere of the olfactory epithelium projects to the medial, G_{olf}-non-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

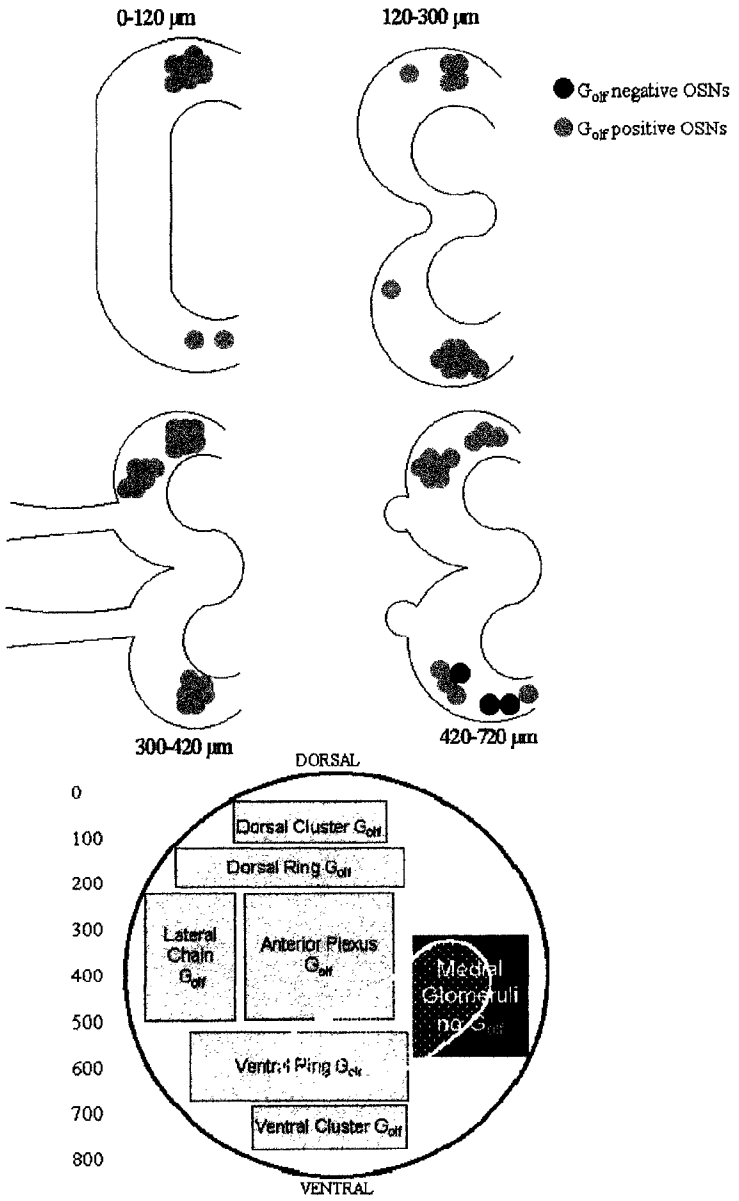


Figure 13. Preparation M2: The ventral hemisphere of the olfactory epithelium projects to the medial, G_{olf}-non-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

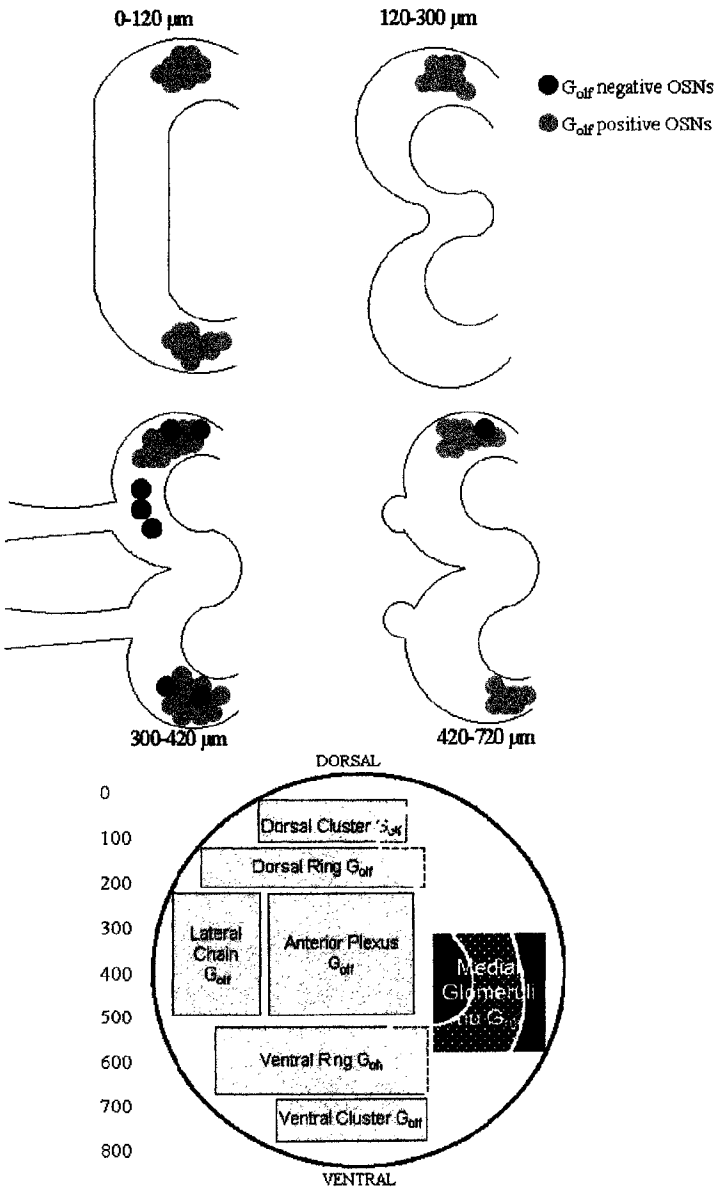


Figure 14. Preparation M3: The ventral hemisphere of the olfactory epithelium projects to the medial, G_{olf}-non-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

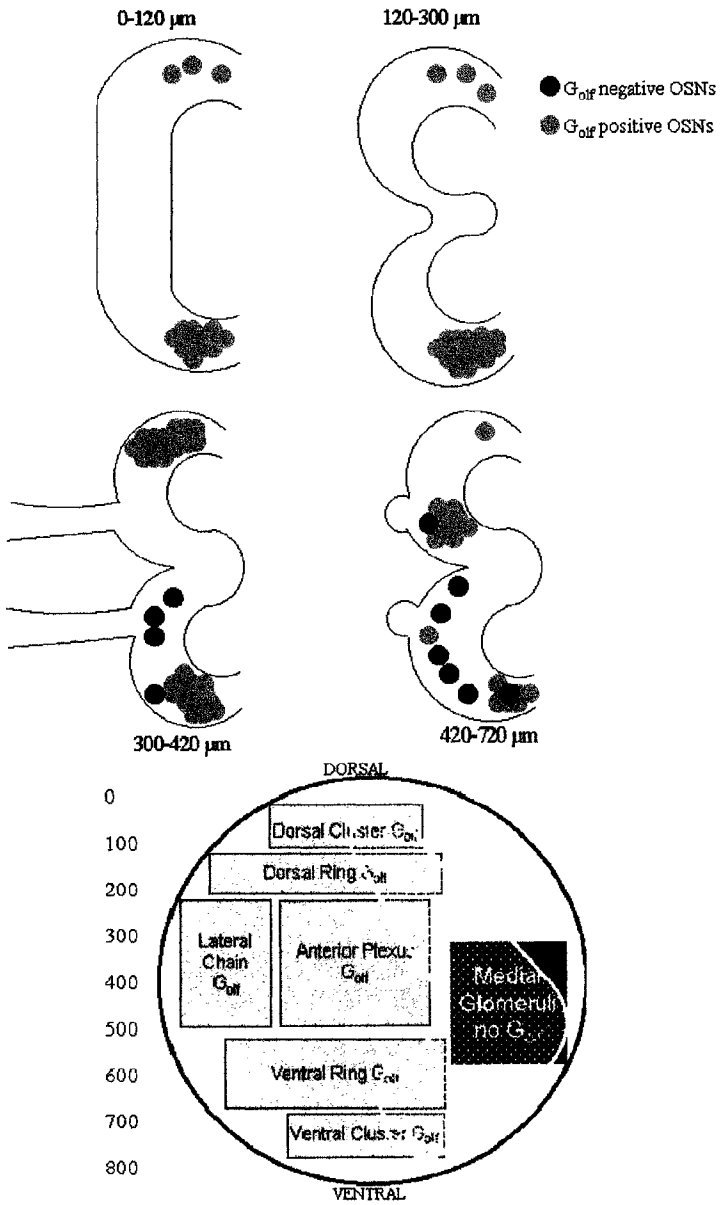


Figure 15. Preparation M4: The ventral hemisphere of the olfactory epithelium projects to the medial, G_{olf}-non-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

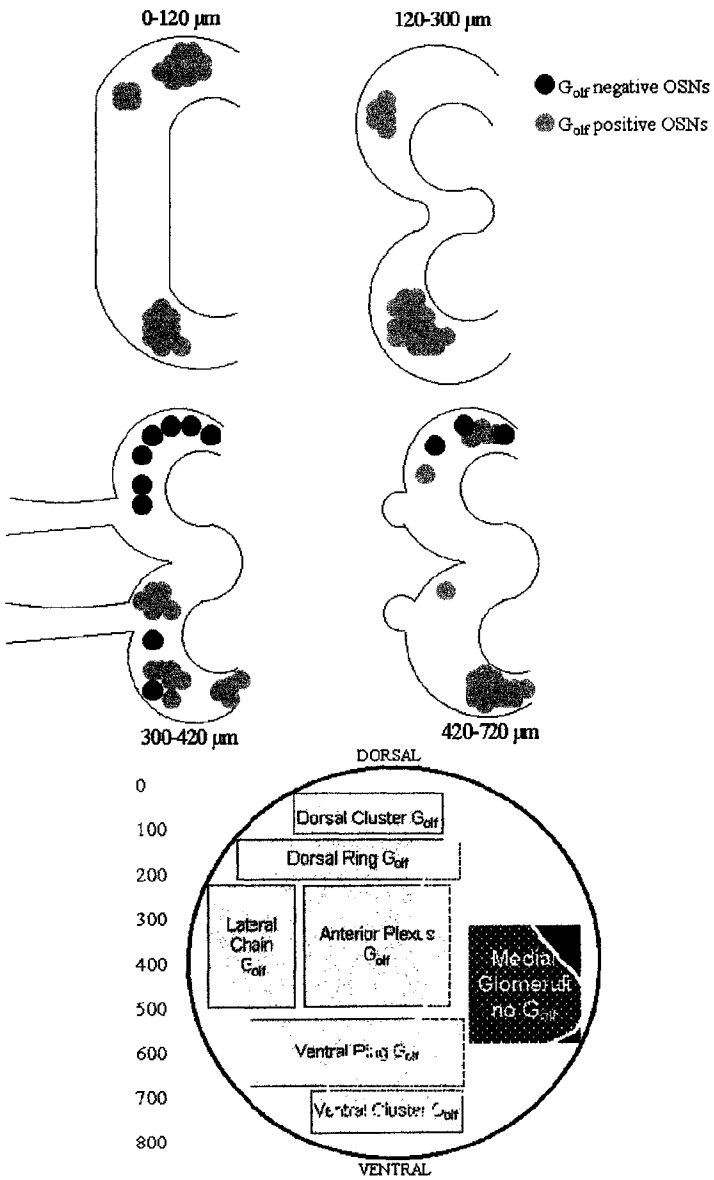


Figure 16. Preparation M5: The ventral hemisphere of the olfactory epithelium projects to the medial, G_{olf}-non-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

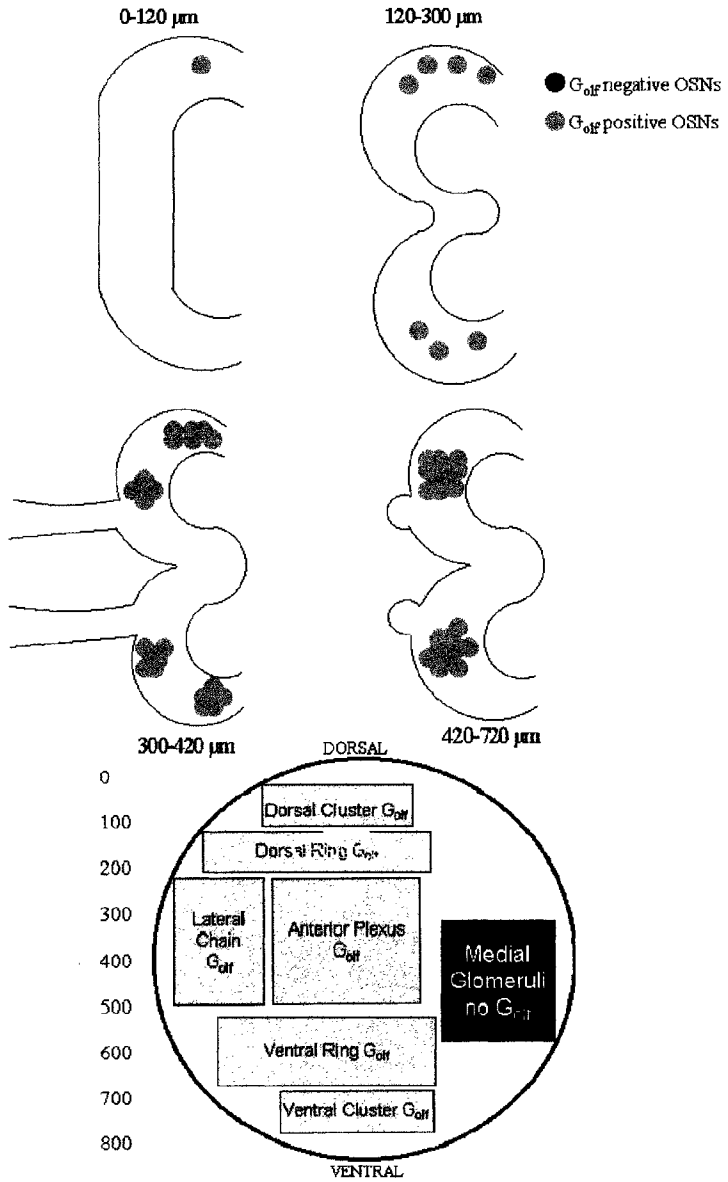


Figure 17. Preparation NM1: All depths of the peripheral olfactory organ project to the non-medial, G_{olf}-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

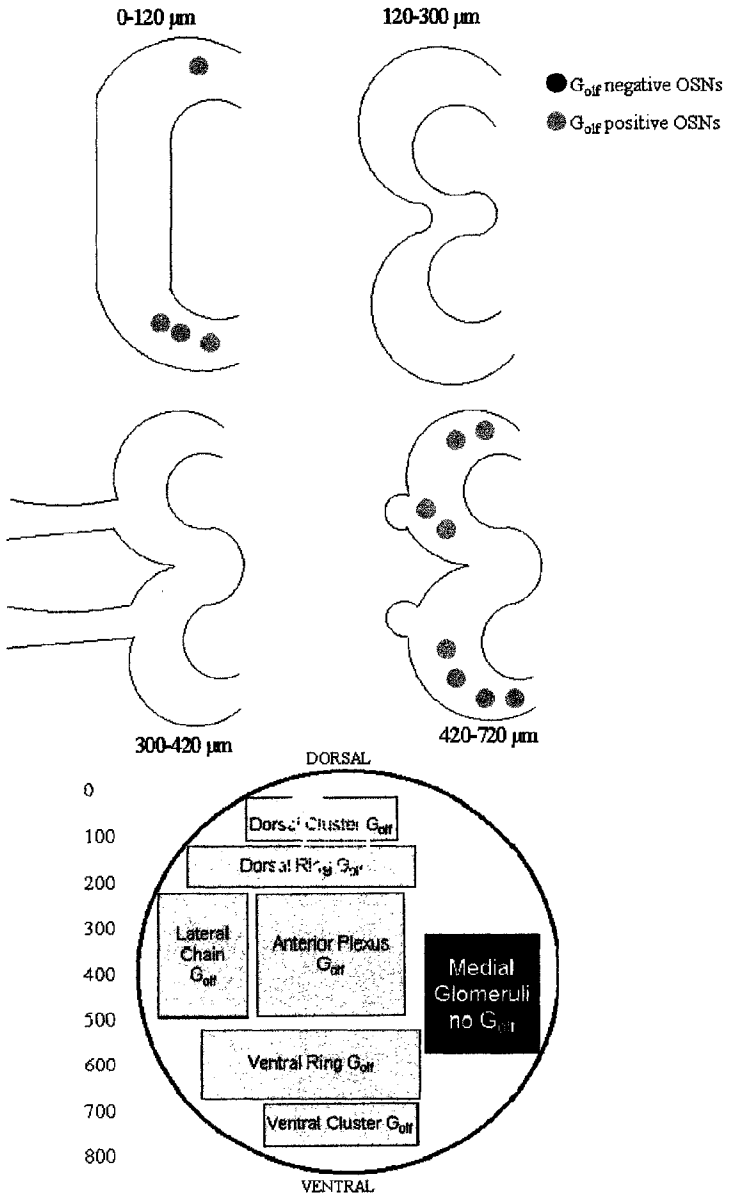


Figure 18. Preparation NM2: The 0-120 µm depth and the 420-720 µm depth of the peripheral olfactory organ project to the non-medial, G_{olf}-IR glomeruli.
Hatched white area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}-IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

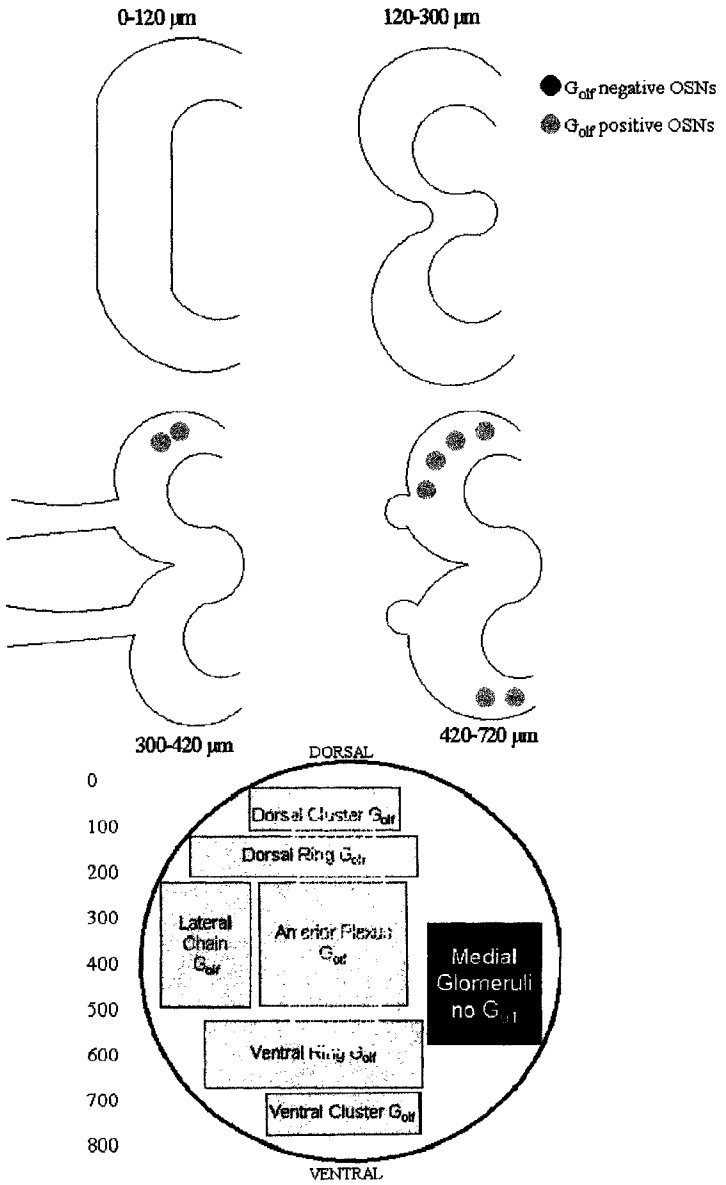


Figure 19. Preparation NM3: The 300-720 µm depths of the peripheral olfactory organ project to the non-medial, G_{olf}-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

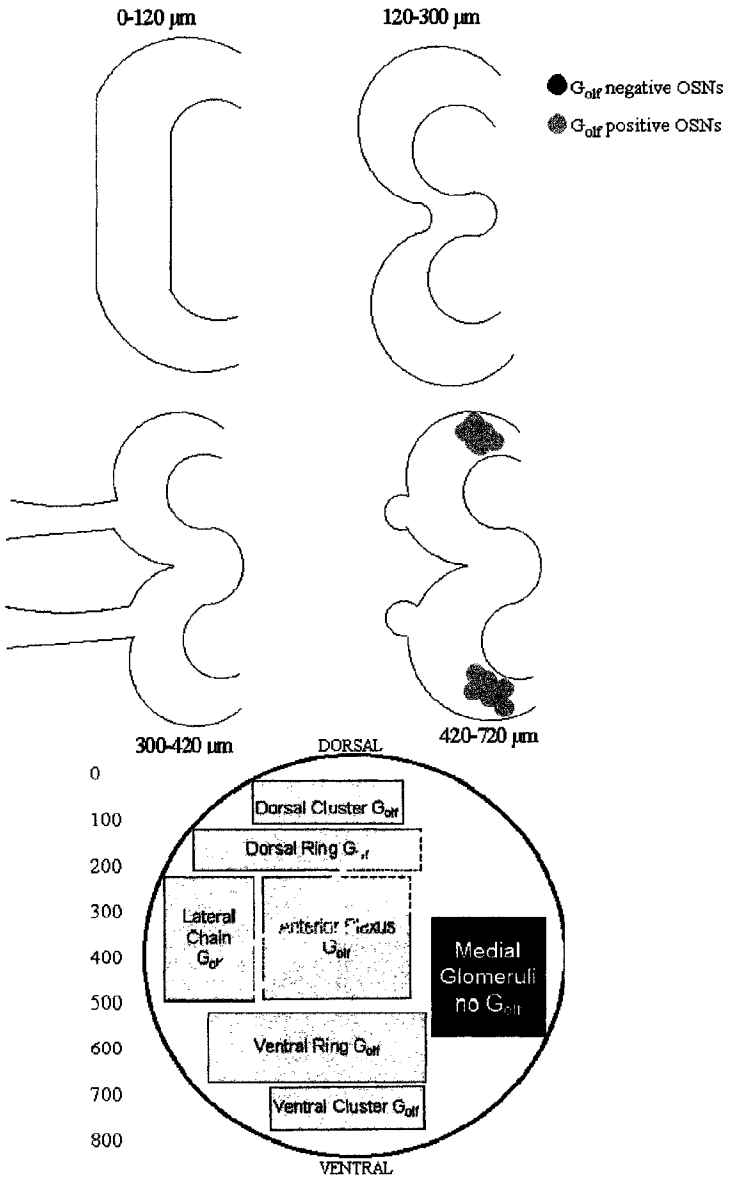


Figure 20. Preparation NM4: The 420-720 µm depth of the peripheral olfactory organ projects to the non-medial, G_{olf}-IR glomeruli. *Hatched white* area in the olfactory bulb indicates the shape of the dextran injection. *Grey* indicates G_{olf}- IR glomeruli and OSNs in the olfactory epithelium. *Black* indicates G_{olf}-non-IR glomeruli and OSNs in the olfactory epithelium. The spatial distribution of OSNs is represented in these templates as a compilation of OSNs at the designated depths in the olfactory epithelium. The dots placed in the olfactory epithelium represent OSN clusters; the position with respect to the basal and apical surface of the olfactory epithelium is irrelevant in these schematic drawings.

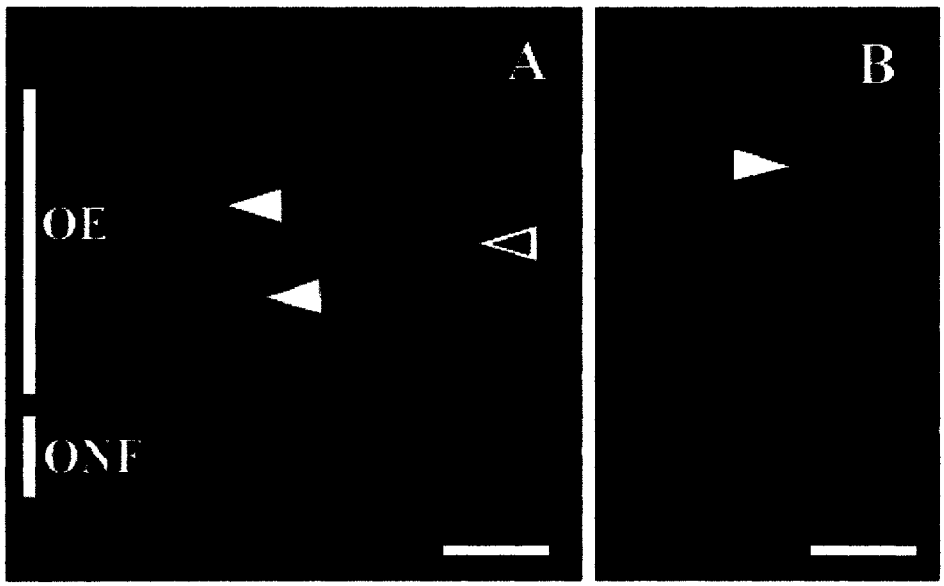


Figure 21. Olfactory epithelium containing dextran back-filled OSNs.
Dextran labeled OSNs back-filled from the olfactory bulb are either G_{olf}-IR (filled arrowheads) or G_{olf}-non-IR (empty arrow heads). G_{olf}-IR OSNs are indicated by a green interior (dextran back-filling) and a red outline of the cell membrane (G_{olf}-IR); G_{olf}- non-IR OSNs are indicated by a green interior only.
(A) Shows two G_{olf}-IR OSNs on the left, and one G_{olf}-non-IR OSN on the right
The olfactory epithelium (OE) and the olfactory nerve fasicles (ONF) are displayed.
(B) Shows one G_{olf}-IR OSN.
Scale bar is 25 μm.

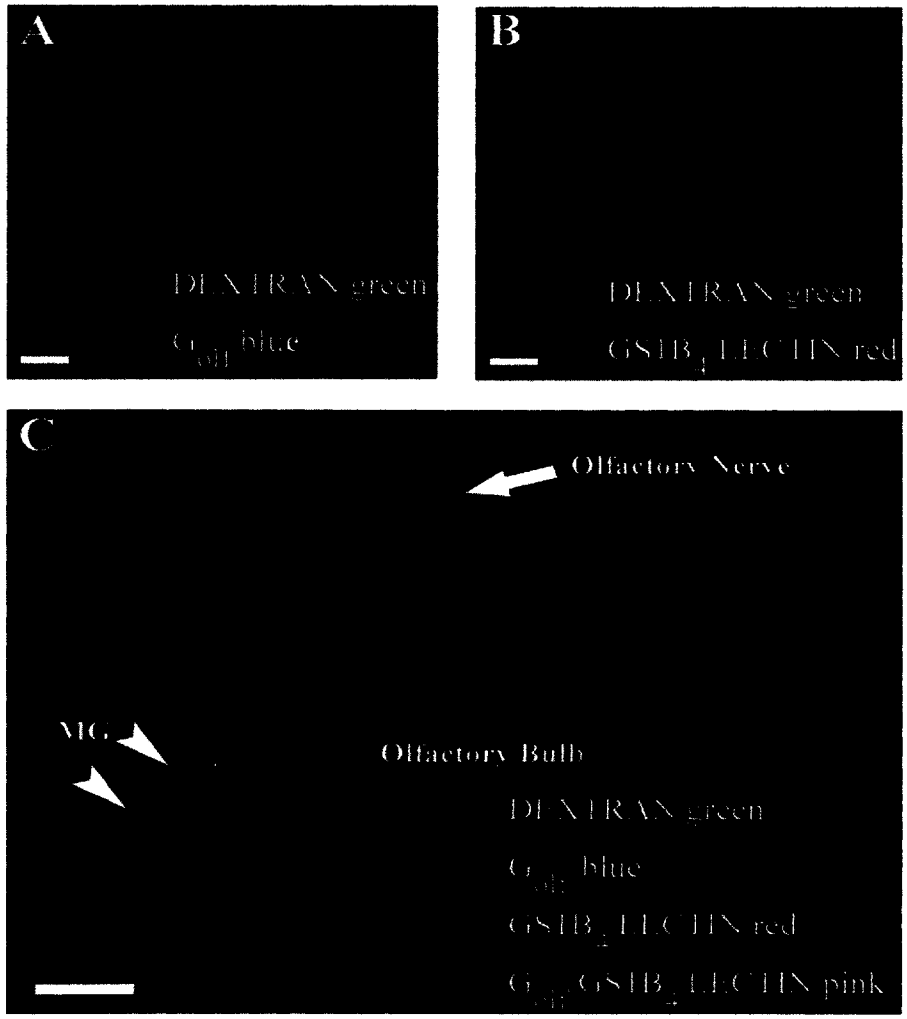


Figure 22. The co-localization of a medial dextran injection with respect to G_{olf}-IR glomeruli. This is a confocal image of a horizontal section from the mid region of the olfactory bulb. Scale bar in each panel is 100 μm.
(A) Shows the labeling of dextran injected medial G_{olf}-non-IR glomeruli (green) and of medial G_{olf}-non-IR glomeruli (blue; G_{olf} immunocytochemistry); this indicates that only medial G_{olf}-non-IR glomeruli are labeled with dextran.
(B) Shows the doulble labeling of dextran injected medial G_{olf}-non-IR glomeruli and the labeling of all glomerular territories in red ($GS1B_4$ lectin labeling); this indicates the doulble label of dextran into the medial glomeruli (yellow hue).
(C) Shows panels A and B merged. The medial glomeruli (MG, filled arrowheads) are G_{olf}-non-IR, shown in red ($GS1B_4$ lectin labeling), overlapped with the green dextran injection. The double labeled medial glomeruli (green dextran injection overlapped with the red $GS1B_4$ lectin) displays a yellow colour. The anterior and lateral glomeruli (pink) are G_{olf}-IR. These anterior and lateral glomeruli are double labeled with $GS1B_4$ and are G_{olf}-IR. This shows the dextran injection is specifically in the medial glomeruli, which are G_{olf}- non-IR.

2.4 Discussion

This study displays a topographic projection from the peripheral olfactory organ to a discrete glomerular territory in a fish species. This is a significant finding, given that previous studies have failed to show this in any fish species. Previous studies have indicated a model of teleost fish olfaction whereby topographic projections between the olfactory bulb and olfactory epithelium is absent (Hara and Zhang, 1996). This finding in the primary olfactory pathway of the larval sea lamprey parallels the spatial organization of OSNs in mammals (Juilfs et al., 1997; Mombaerts, 1999; Ressler et al., 1993; Schoenfeld et al., 1994; Vassar et al., 1993). Hence, this study may provide new insight into the currently accepted concepts in fish olfactory biology. The fact that the sea lamprey, a jawless vertebrate, may organize olfactory information in a different fashion than teleost fish species may be indicative of its unique evolutionary history.

In this study with the larval sea lamprey, a jawless agnathan, a topographic projection between the olfactory epithelium and the olfactory bulb has been indicated. This study is the first to show that a subset of G_{olf}-non-IR OSNs, localized exclusively in the ventral hemisphere of the peripheral olfactory organ, converges onto the medial glomerular territories in the olfactory bulb in any fish species. G_{olf}-IR OSNs throughout the peripheral olfactory organ projected into the remaining glomerular territories. Other than one study in a teleost (common carp, *Cyprinus carpio*) that indicated a spatial projection of specific regions of the OE to the olfactory bulb (Sheldon, 1912), this finding in the non-teleost sea lamprey is novel in fish. In carp, the medial bundle of the olfactory nerve is derived from the rostral lamellae and the lateral bundle is derived from the caudal lamellae (Satou et al., 1983). Therefore, a spatial division of OSNs, as defined by

differing signal transduction cascades, is present in the olfactory epithelium and the olfactory bulb.

The expression of alternate G-proteins in OSN subtypes seems to be a fundamental principle underlying the olfactory system of vertebrates. In the catfish (*Ictalurus punctatus*), ciliated G_{olf}-IR OSNs projected to medial and ventral regions of the olfactory bulb; and microvillous OSNs that express $G_{\alpha o}$ and $G_{\alpha q/11}$ projected to the dorsal region of the olfactory bulb (Hansen et al., 2003). In the lamprey (*Lampetra fluviatilis*), OSNs with differeing levels of calretinin-IR extend to particular glomerular locations (Pombal et al., 2002). In the mammalian VNO, receptor neurons that express $G_{i\alpha 2}$ and $G_{o\alpha}$ projected to segregated glomeruli in the accessory olfactory bulb (Jia and Halpern, 1996). The existence of distinct sub-populations of OSNs with alternate G protein expression, which project to discrete glomelular units is consistent with the findings of this study.

The presence of the medial glomeruli in the larval sea lamprey which lack the expression of G_{olf} and project to a spatially conserved subset of OSNs in the olfactory epithelium suggests an indepenedent medial pathway. Previous studies have shown that the medial area of the olfactory bulb is associated with the perception of reproductive sex pheromones in many teleosts (Stacey and Kyle, 1983; Kyle et al., 1987; von Rekowski and Zippel, 1993; Friedrich and Korsching, 1997; Hara and Zhang, 1996; Laberge and Hara, 2003; Weltzien et al., 2003). This medial olfactory system in teleosts may "functionally" correspond to the accessory olfactory system (VNO) in higher vertebrates (Satou, 1990); it has been suggested that a vomeronasal system is present in some fishes, but in a form not yet recognized (Eisthen, 1992). In the goldfish, the medial olfactory

tract, which is responsive to reproductive sex pheromones may be a homologue of the tetrapod VNO, and the lateral olfactory tract, to the tetrapod main olfactory system (Dulka, 1993). Therefore, this G_{olf}-non-IR OSN subset that exists in the larval sea lamprey, which likely implements an alternate signal transduction cascade, may be functionally similar to the tetrapod VNO. Further studies are required to determine the functional responses of these medial glomeruli in the sea lamprey.

The spatial segregation of a subset of G_{olf}-non-IR OSNs in the peripheral olfactory organ and the topographic projection onto a discrete glomerular territory is a novel finding. Randomly dispersed OSNs in the olfactory epithelium that possess distinct G protein-IR have been shown to converge onto discrete glomeruli in teleost fish (Hansen et al., 2003); however, these biochemically distinct OSNs have not localized to a specific region of the peripheral olfactory organ as in the larval sea lamprey. It appears that in the rainbow trout (*Oncorhychus mykiss*), Channel Catfish (*Ictalurus punctatus*), the crucian carp (*Carassius carassius L.*), Atlantic salmon (*Salmo salar*) the zebrafish (*Danio rerio*), the presence of point-to-point topography is lacking (see discussion that follows). In the rainbow trout (*Oncorhychus mykiss*), labeled OSNs were widely dispersed in the olfactory epithelium upon injection into discrete glomeruli (Riddle and Oakley, 1991). In the channel catfish (*Ictalurus punctatus*), morphologically distinct OSNs, each expressing unique G proteins, are widely dispersed in the olfactory epithelium and project to spatially segregated areas of the olfactory bulb (Hansen et al., 2003). Also, *in situ* hybridization studies in catfish has shown that neurons expressing specific receptors are distributed randomly within the olfactory epithelium (Ngai et al., 1993a,b). These two studies indicated the absence of any specific topography in the

peripheral olfactory organ. Likewise, in the crucian carp (*Carassius carassius L.*), the projection of morphologically distinct OSNs that are randomly distributed throughout the olfactory epithelium project to discrete parts of the olfactory bulb. In both the Atlantic salmon (*Salmo salar*) and rainbow trout (*Oncorhyncus mykiss*), OSNs that responded to specific odourants are dispersed in the olfactory epithelium and converge onto common glomeruli located in segregated regions of the olfactory bulb (Hara and Zhang, 1996). In these two salmonid fishes, the OSNs responsive to amino acids and taurocholic acid were found to be randomly distributed throughout the olfactory epithelium, but they specifically project to the lateroposterior and the medial areas of the olfactory bulb, respectively (Hara and Zhang, 1996). In the zebrafish (*Danio rerio*), the positions of OSNs in the olfactory epithelial surface also does not predict glomerular specificity (Baier et al., 1994). The projection of G_{olf}-non-IR OSNs located in the ventral hemisphere of the olfactory epithelium to the medial glomeruli is a significant and novel finding in the larval sea lamprey; this is likely indicative of the sea lamprey's divergent evolutionary history from teleosts.

The evidence for discrete point-to-point topographic projections lies in mammals and invertebrates (Christensen et al., 1995, Juilfs et al., 1997; Mombaerts, 1999; Ressler et al., 1993; Schoenfeld et al., 1994; Vassar et al., 1993). In invertebrates such as the sphinx moth (*Manduca sexta*), distinct populations of olfactory receptor axons in the antennal lobe project to specific glomeruli (Christensen et al., 1995). Axons of sex-pheromone-selective receptor cells in the male-specific type-I trichoid sensilla project exclusively to the sexually dimorphic macroglomular complex (Christensen et al., 1995). Studies in mammals indicate the presence of specific, sharply bounded zones in the

olfactory epithelium that each express specific olfactory receptor genes or G proteins which project to distinct glomeruli in the olfactory bulb (see specific details in the introduction). Likewise, in this study of the larval sea lamprey, OSNs localized in a specific region of the olfactory epithelium have been shown to project to specific glomeruli in the olfactory bulb. Although this study in the larval sea lamprey has not shown sharply bounded zones in the olfactory epithelium as is present in mammals, the projection from the medial glomeruli does indicate the presence of a single point-to-point projection in an agnathan. A clear boundry dividing the olfactory epithelium based on OSN subtypes that differentially express G_{olf} may exist in the larval sea lamprey; however, the neuronal tract tracer technique implemented in this study is limited. In mammals, the presence of roughly bounded zones of epithelium projecting to distinct glomeruli was detected using neuronal tract tracers (Schoenfeld, et al., 1994). However, the presence of sharply bounded zones of epithelium projecting to specific glomeruli was detected using monoclonal antibodies that selectively label specific cell surface proteins (Mori et al., 1999; Yoshihara et al., 1997; Schwob and Gottlieb, 1988). This differentially labeled specific subsets of OSNs in the olfactory epithelial sheet and their specific projections onto distinct glomeruli (Mori et al., 1999; Yoshihara et al., 1997; Schwob and Gottlieb, 1988). Likewise, in the sea lamprey, antibodies against specific cell surface markers and olfactory receptors could be implemented to detect if sharply bounded zones of the epithelium does exist. Hence, the sea lamprey, mammals, and invertebrates do implement point-to-point topographic projections in their olfactory systems. The presence of point to point topography in the olfactory system seems to be an evolutionarily conserved principle in these phylogenically divergent sensory systems.

In conclusion, a subset of G_{olf}-non-IR OSNs has been identified in the ventral hemisphere of the olfactory epithelium that extends axons exclusively to the medial, G_{olf}-non-IR glomeruli. This implies the presence of a functionally distinct medial pathway that implements an alternate G protein for signal transduction. Since the medial olfactory pathway and medial olfactory tract in many fishes is known to selectively process pheromonal information, these G_{olf}-non-IR OSNs in the ventral hemiphere of the olfactory epithelium may be specialist OSNs for the detection of pheromones. Hence, this study indicates that the differential spatial expression of alternate G proteins within the primary olfactory pathway in vertebrates may have evolved before gnathostome radiation or in parallel.

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CHAPTER 3: The *in vitro* biosynthesis of novel 5β reduced steroids in the testis of the round goby, *Neogobius melanostomus*

3.1 Introduction

The parental male round goby (*Neogobius melanostomas),* a bottom-dwelling teleost fish, maintains and guards a nest into which many females deposit eggs (Wickett and Corkum, 1998; MacInnis and Corkum, 2000). It has been hypothesized that male round gobies release a pheromone (or a mixture of pheromones) to attract reproductive females to their nests, and that this pheromone is a steroid originating in the testes (Murphy et al., 2001). Strong evidence to support this hypothesis comes from studies on another species from the subfamily Gobiidae, the black goby, *Gobius jozo* (=*G. niger*). Male black gobies have been shown to emit a pheromone that attracts females to their nests (Mozzi, 1968). Colombo et al. (1977; 1982) have shown that the testes of black gobies contain prominent Leydig (steroid-secreting) cells, concentrated in the region where the testis is suspended from the body wall by lengthwise mesenteries known as mesorchia, and hence termed the 'mesorchial gland'. Colombo et al. (1970; 1977) showed that the mesorchial gland was capable of transforming radioactive pregnenolone into predominantly conjugated and 5β-reduced steroids. The most prevalent of these was 17-oxo-5β-androstan-3α-yl glucuronide (etiocholanolone glucuronide; ETIO-g). This steroid was also shown to act as an attractant to gravid females (Colombo et al., 1980).

Previous studies of the round goby support the view that this species also releases steroids with pheromonal properties. Murphy et al. (2001) examined olfactory epithelial (electro-olfactogram, EOG) responsiveness of the round goby to over 100 steroids and prostaglandins. The prostaglandins were all inactive, however, 19 steroids elicited

responses. Cross-adaptation studies with these steroids, revealed that there were four classes of olfactory receptors that Murphy et al. (2001) named: E1, E2-3-g, ETIO and DHEA-s, after the steroids that gave the highest response to each class of receptor (estrone [E1], 17β-estradiol 3-glucuronide [E2-3g], etiocholanolone [ETIO] and 17-oxo-androst-5-en-3β-yl sulfate [dehydroepiandrosterone sulfate; DHEA-s]. Murphy et al. (2001) discovered that males markedly increased their gill ventilation rate in response to steroids that acted on the E1, E2-3g and ETIO receptors, while females only responded to steroids that acted on the ETIO receptor. In a subsequent study (Murphy and Stacey, 2002), females responded to E1 and E2-3g following treatment with methyl testosterone, suggesting that these classes of receptors were involved in female-to-male communication.

The aims of the present study were: firstly, to establish whether the testis of the round goby also contains a homolog of the mesorchial gland; secondly, to determine whether this gland was able to convert radioactive androstenedione (androst-4-ene-3,20-dione; Ad) and 17-hydroxypregn-4-ene-3,20-dione (17-P) into other steroids *in vitro*; thirdly, to determine the identity of these steroids. Due to time constraints, identification studies were mainly restricted to the steroids that were formed from tritiated Ad. Underlying this decision was the fact that there are a relatively small number of other steroids into which Ad (a C_{19} steroid) can be converted, compared with when pregnenolone or 17-P (both C_{21} steroids) are used as precursors. There is a risk to this strategy in that the pheromone may actually be a C_{21} steroid or even a 5-ene steroid. However, although Murphy et al. (2001) have shown that round gobies gave an EOG response to six 5β-reduced C_{21} steroids, this response was much weaker than that to

ETIO and could also be significantly reduced by cross-adaptation with ETIO. The work on the black goby (see above) also strongly implicates 5β-reduced C_{19} steroids as pheromones.

The importance of this work lies in the fact that the round goby has invaded the Great Lakes of North America and is posing a threat to native fish species (Corkum et al., 1998). If a male sex pheromone can be identified in this species, it may possibly be useful for trapping females and/or disrupting their spawning.

3.2 Materials and methods

3.2.1 Experimental animals

Round gobies were obtained from the Detroit River (Windsor, ON, Canada) by angling and were maintained at a water temperature of 18 to 20°C in dechlorinated tap water. Spermiating males were captured in June 2002 (fish I and IV) or July 2003 (fish II to V). Spermiating males were identified by swollen cheeks and black coloration (MacInnis and Corkum, 2000). Furthermore, the presence of active sperm and histological examination of the testis was completed in this study to confirm reproductive maturity. The fish were anaesthetized with MS222, killed and the testes dissected and placed in ice-cold Leibowitz L15 medium (Sigma-Aldrich, USA).

In July 2003, two males (fish II and III) were injected with 10 I.U./g body weight of human chorionic gonadotropin (HCG) – dissolved in saline at a concentration of 250 I.U./ml. The testes were dissected out 12 hours after the HCG injection. These HCG injections were performed to determine if it would have any noticeable effect on the types and relative amounts of steroids that were produced.

3.2.2 Histological prepartions

Following deep anesthesia of two spermiating round gobies in MS 222, the testes were removed and immediately immersed into Karnovsky fixative (0.075M cacodylate buffer, 2.2% paraformaldehyde, 2% glutaraldehyde) overnight, then cut into 1 mm slices by hand, on the following morning, and immersed into fresh fixative for 2 hours. The fixed tissue slices were post-fixed in 1% osmium tetroxide (in 0.075M cacodylate buffer), dehydrated through an ascending series of ice-cold ethanol, passed through propylene oxide and embedded into epoxy resin. Serial semi-thin (1μm) sections were taken on an ultramicrotome, stained with 1% toluidine blue (in 1% sodium tetraborate), viewed by brightfield microscopy and photographed on an Zeiss Axioscope 2 FS.

Testes from a further ten round gobies were prepared into paraffin, sectioned serially at 3 μm, and were also examined. This procedure, which was less time consuming and less technically demanding than the plastic method, confirmed the same cellular organization in all of the animals that were examined. Here, we present the data obtained from the plastic sections because of clarity and high resolution by brightfield light microscopy.

3.2.3 *In vitro* incubation of testes with tritiated precursors

Five pairs of testes (fish I to V) were incubated with [1,2,6,7-^{3}H] androst-4-ene-3,17-dione (including those from the two fish that had been injected with HCG) and one pair of testes (fish VI) with 17-hydroxy[1,2,6,7-^{3}H] progesterone. Both radiochemicals were purchased from Amersham Biosciences. The diffuse spatial arrangement of the Leydig

cells in the round goby testis, as seen at high power in the histological preparations, precluded selection of regions that were rich in Leydig cells (cf. mesorchial gland of the black goby: Columbo, 1970, 1977, 1982) for *in vitro* incubation. The testes were thus dissected from the fish, separated and then finely minced, suspended in ice-cold Leibowitz L15 medium (Sigma) and transported on ice from the University of Windsor to Michigan State University (ca. 3 h). Here, the bits of tissue were distributed to fresh 50 ml plastic tubes containing 10 ml Leibowitz L15 medium and 10 to 50 μCi tritiated Ad or 17-P. The tubes were laid on their side and gently agitated at 16 °C for a further 6 h (Kime and Scott, 1993). At the end of the incubation period, the medium was filtered and then passed through a Sep-Pak C-18 cartridge (Waters Chromatography, Millipore, Milford, MA, USA). This was washed with 5 ml distilled water and then eluted with 5 ml methanol. The extract was stored at –20 °C.

3.2.4 Separation of tritiated metabolites from reproductive male round goby by High-Performance Liquid Chromatography (HPLC)

The methanol extracts from the Sep-Pak C-18 cartridges were dried down either under a stream of nitrogen at 45°C or in a rotary evaporator, mixed with 10 μg each of Ad and 11-oxo-T, reconstituted in 1 ml acetonitrile/water/trifluoroacetic acid (28/72/0.01; v/v/v) and then loaded onto an analytical reverse-phase HPLC column (Rainin Dynamax Microsorb; 5μm octadecylsilane; 4.6mm x 25 cm; fitted with a 1.5 cm guard module). Two pumps were used to deliver solvents through the column at a rate of 0.5 ml/min. Solvent A was 0.01% trifluoroacetic acid (TFA) in distilled water and solvent B was 70% acetonitrile and 0.01% TFA in distilled water: 0-10 min, 28.6% B; 10-60 min, 28.6-100% B; 60-80 min, 100% B; 80-82 min, 100-28.6% B; 82-100 min, 28.6% B. One minute fractions were collected between 20 and 70 minutes. A volume of 5μl from each

HPLC fraction was then counted in a scintillation counter and fractions that formed distinct peaks on HPLC were saved for identification studies. Part of the identification criteria for metabolites involved the position that they eluted on HPLC. This was established for the synthetic standards by running 10 or 20 μg of each on the HPLC column under the same running conditions as above and monitoring the eluate with a diode-array detector. Steroids with a 4-ene configuration (e.g. Ad and 11-oxo-T) gave a strong peak of UV adsorption at ca. 248 nm. Steroids with a C=O group (or groups) gave a much weaker, but nevertheless distinct, peak of UV adsorption at ca. 200 nm.

3.2.5 Identification of steroids: Thin-Layer Chromatography (TLC) of HPLC Fractions

The standard synthetic steroids used in this study (Table 4) were purchased from Sigma Chemical Company or Steraloids. I tried to obtain all the steroids into which Ad might be converted via 'well-established' biosynthetic pathways. Using this criterion, we might expect there to be metabolites with either androst-4-ene, 5α-androstan or 5β-androstan configurations. In the 5α- and 5β-reduced steroids, we might expect to find either a 3-oxo, a 3α-hydroxyl or a 3β-hydroxyl group. In the androst-4-ene steroids, we would expect to find only a 3-oxo group. We would not expect to find 3-reduced androst-4-ene steroids as, to our knowledge, direct reduction of the 3-oxo group of 4-ene steroids has not been demonstrated in any vertebrate (although such steroids may be formed when 5-ene steroids are used as intermediates; Inaba et al., 1966). We might expect the 17-oxo group to be either retained or reduced to form a 17β-hydroxyl (but not 17α-hydroxyl) group. We might expect the 11-carbon to remain as it is or to be 11-oxygenated to form an 11β-hydroxyl (but not 11α-hydroxyl) group and then also further

oxidized to form an 11-oxo group. All these possible combinations are shown in Table 4 (including eight steroids that we were unable to obtain).

There is always the possibility that Ad will be converted to C_{18} (estrogen-like) steroids. However, we have not included these in Table 4 as estrogen formation by testes has not been reported in teleosts (Fostier et al., 1983). No estrogen formation was found in previous studies on the testes of black goby (Colombo et al., 1977) or urohaze-goby *Glossogobius olivaceus* (Asahina et al., 1985).

We might also expect that any steroid in Table 4 that has either a 3α-oxo, 3β-oxo or 17β-oxo group could be glucuronidated or sulfated. Only four examples (that are referred to within the present chapter) are shown in Table 4. Identification of conjugated steroids normally involves their deconjugation through either enzyme hydrolysis (which works on glucuronides and some sulfates) or acid solvolysis (which works on sulfates only) (Scott and Vermeirssen, 1994). Only the latter method was applied in the present study (see below).

All steroids were dissolved in ethanol at a concentration of 500 μg/ml. At the outset of the identification work, 10 μg of each steroid was combined with 10 μg testosterone (T), spotted onto separate lanes of the TLC plates and developed with chloroform/methanol (50/2, v/v). The positions of the androst-4-ene steroids (e.g. Ad, T, 11-oxo-T) were detected by shining a UV lamp at the plate. Steroids with reactive hydroxyl groups (e.g. 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-ETIO) were detected by spraying with a solution of phosphomolybic acid in ethanol (10% w/v) and heating the plate for 5 min at 150°C. Steroids with no reactive hydroxyl groups (e.g. 5β-androstane-3,11,17-trione) were detected by spraying with concentrated sulfuric acid in

methanol (10% v/v) and heating the plate for 5 min at 150°C. After the bands were revealed, their distance to the origin was measured to the nearest millimeter. The relative position of each steroid in relation to that of T was then calculated. This ratio (R_T) formed the basis of a table (Table 4) against which the relative elution positions of radioactive bands could subsequently be compared.

To determine the identity of the radioactive steroids in the various peaks, ca. 20,000 dpm was mixed with 10 μg T, dried down, reconstituted in 30 μl ethyl acetate and spotted and run on TLC as described above. The position of T was marked with a pencil and each lane then divided into 5 mm strips that were scraped off, mixed directly with 7 ml scintillation fluid and counted. The position of the radioactivity relative to that of T was then compared with the R_T values in Table 4. By doing this, it was possible to narrow down the range of possible matching standards for each HPLC peak. A more precise match was obtained by mixing 20,000 dpm of radioactive steroid with 10 μg each of those steroids that had the most similar R_T values and then running them together on TLC. After the bands had been revealed, they were marked and scraped off, as was 2 x 0.5 cm of silica gel from either side of the bands. All the scrapes were mixed with scintillation fluid and counted. In any situation where the radioactivity was > 90% associated with a band, microchemical studies were then carried out to further establish the co-identity of radioactive metabolite and standard steroid.

Table 4: Standard synthetic steroids against which the tritiated metabolites were compared. R_T is the relative position of each steroid in relation to testosterone (T) when run on TLC. HPLC elution times were only determined for steroids of interest.

Steroid[a]	Common name	R_T[b]	HPLC elution (minutes)
Available steroids:			
3α,17β-dihydroxy-5β-androstan-11-one		0.12	
5α-androstane-3β,11β,17β-triol		0.14	
11β,17β-dihydroxyandrost-4-en-3-one	11β-OH testosterone	0.16	40
3β,11β-dihydroxy-5α-androstan-11-one		0.27	
17β-hydroxyandrost-4-ene-3,11-dione	11-oxo-testosterone (11-oxo-T)[c]	0.4	40
3β,11β-dihydroxy-5α-androstan-17one		0.44	
3α,11β-dihydroxy-5β-androstan-17-one		0.45	
5β-androstane-3α,17β-diol		0.48	
17β-hydroxy-5β-androstane-3,11-dione		0.48	45
3α,11β-dihydroxy-5α-androstan-17-one		0.53	
3β,11β-dihydroxy-5β-androstan-17-one		0.57	
17β-hydroxy-5α-androstane-3,11-dione		0.58	
3α-hydroxy-5β-androstane-11,17-dione	11-oxo-etiocholanolone (11-oxo-ETIO)	0.67	47
11β-hydroxyandrost-4-ene-3,17-dione	11β-OH androstenedione (11β-OH-Ad)	0.72	43
5α-androstane-3α,17β-diol		0.72	
3α-hydroxy-5α-androstane-11,17-dione		0.77	
5α-androstane-3β,17β-diol		0.78	
3β-hydroxy-5α-androstane-11,17-dione		0.79	
5β-androstane-3β,17β-diol		0.86	
17β-hydroxyandrost-4-ene-3-one	Testosterone (T)	1.0	52
3α-hydroxy-5β-androstan-17-one	Etiocholanolone (ETIO)	1.0	60
11β-hydroxy-5α-androstane-3,17-dione		1.03	
11β-hydroxy-5β-androstane-3,17-dione		1.06	
17β-hydroxy-5β-androstan-3-one		1.12	
3β-hydroxy-5α-androstan-17-one		1.14	
3α-hydroxy-5α-androstan-17-one		1.20	
17β-hydroxy-5α-androstan-3-one		1.28	
3β-hydroxy-5β-androstan-17-one		1.28	
5β-androstane-3,11,17-trione		1.33	48
androst-4-ene-3,11,17-trione	11-oxo-androstenedione; androstenetrione	1.38	45
5α-androstane-3,11,17-trione		1.49	47
androst-4-ene-3,17-dione	Androstenedione (Ad)	1.70	55
5β-androstane-3,17-dione		1.82	

5α-androstane-3,17-dione		1.94	
Conjugated steroids:			
17-oxo-5β-androstan-3α-yl glucuronide	Etiocholanolone glucuronide (ETIOg)	0	44
17-oxo-5β-androstan-3α-yl sulfate	Etiocholanolone sulfate (ETIOs)	0	37
11,17-dioxo-5β-androstan-3α-yl glucuronide	11-oxo-etiocholanolone glucuronide (11-oxo-ETIOg)	0	38
11,17-dioxo-5β-androstan-3α-yl sulfate	11-oxo-etiocholanolone sulfate (11-oxo-ETIOs)	0	30
Steroids (in same range as above) that were not available for testing:			
5α-androstane-3α,11β,17β-triol			
5β-androstane-3β,11β,17β-triol			
5β-androstane-3α,11β,17β-triol			
3α,17β-dihydroxy-5α-androstan-3-one			
11β,17β-dihydroxy-5α-androstan-3-one			
3β,17β-dihydroxy-5β-androstan-11-one			
11β,17β-dihydroxy-5β-androstan-3-one			
3β-hydroxy-5β-androstane-11,17-dione			

[a] the nomenclature of steroids in this chapter follows that recommended by IUPAC (Kime, 1995; www.chem.qmul.ac.uk/iupac/steroid) and not that used by Steraloids Inc. (www.steraloids.com), Sigma-Aldrich (www.sigma-aldrich.com) or Murphy et al. (2001).

[b] the R_T values and HPLC elution times shown in Table 4 are only approximate and are expected (and were found) to differ slightly in response to temperature and exact composition of solvents; however, approximate R_T provided a useful 'first step' in the identification of products from incubation of testis with tritiated Ad.

[c] also known incorrectly as 11-keto-testosterone

3.2.6 Microchemical reactions

Prior to each microchemical reaction, the radioactive metabolite (ca. 20,000 dpm) and the cold standard (10 to 20 μg) were mixed together and evaporated under a stream of nitrogen at 45°C. In some situations, in addition to the above, the radioactive steroid was treated in the absence of cold standard and then mixed with 10 μg of the 'expected' product prior to being run on TLC.

Enzymic oxidation: The steroid was redissolved in 1 ml 0.05M Tris-HCl (pH 9) containing 1.5 mg NAD (Sigma N-1511) and 1.2 mg Hydroxysteroid Dehydrogenase (HSD; Sigma H-8879). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate.

Enzymic reduction: The steroid was redissolved in 1 ml 0.05M Tris-HCl (pH 7.6) containing 3.3 mg HSD (Sigma, H8879) and 2.4 mg NADH (Sigma, N-8179). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate.

Chemical oxidation: The steroid was redissolved in 100 μl glacial acetic acid and 60 μl of chromium trioxide solution (20 mg/ml of distilled water). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate, which was washed twice with 1 ml sodium bicarbonate and twice with 1 ml distilled water before being evaporated.

Acetylation: The steroid was redissolved in 100 μl pyridine and 100 μl acetic anhydride and left overnight. The pyridine and acetic anhydride were removed by drying down with a stream of nitrogen at 45°C. The plates were developed with chloroform/ethanol 50/1 (v/v) to prevent potential acetylated products from migrating too far up the plate.

Acid Solvolysis: Acid solvolysis was carried out to remove sulfate groups from sulfated steroids. The radioactive steroid was dissolved in 5 ml ethyl acetate/trifluoroacetic acid (100 ml/1.4ml, v/v) and incubated overnight at 45 °C. The solvents were removed by drying down under a stream of nitrogen at 45°C.

3.2.7 Recrystallization (performed by Dr. A.P. Scott, The Center for Environment, Fisheries and Aquaculture Sciences, Weymouth, U.K.)

Definitive evidence for the identity of several of the radioactive steroids was obtained by mixing them with 15 to 20 mg of standard steroid and then repeatedly crystallizing them with either acetone/water or ethanol/water. A portion of steroid was removed after each crystallization, dried and carefully weighed and then redissolved in ethanol and counted to determine specific radioactivity (dpm/mg).

3.2.8 DEAE ion exchange protocol (performed by Dr. A.P. Scott, The Center for Environment, Fisheries and Aquaculture Sciences, Weymouth, U.K.)

The column was made from two 1 ml HiTrap™ DEAE FF cartridges (Amersham Biosciences) linked in series. There were two buffer solutions. Solution A consisted of Tris-HCl 0.05M (pH 7.8) made up in a mixture of 1250 ml deionised water and 250 ml ethanol. Solution B consisted of 0.5 M NaCl made up in the same buffer as solution A. The column was equilibrated with solution A at a flow rate of 0.15 ml/min. The test compound (20,000 dpm) was mixed with 10 μg each of free, sulfated and glucuronidated 17,20β-dihydroxypregn-4-en-3-one dissolved in 1 ml of solution A. This was injected on to the column at 0.15ml/min. After 5 ml of solution A had passed through the column, a gradient of 0% solution B to 100% solution B over 10 min was then applied. The

eluate was monitored at 254 nm and 500 µl fractions were collected between 0 and 5 ml and 1 ml fractions between 5 and 15 ml. These were mixed with scintillation fluid and counted.

3.3. Results

3.3.1 Histology

The structure and cellular organization of the testis was the same in all samples that were examined in this study. The testes of the spermiating round goby were, as in many other fish, paired, elongated white-colored organs. When a testis was viewed in a cross-sectional plane, the mesorchial region, located where mesenteries extend to the body wall, was recognized by a deep fold (Figure 23A). Seminiferous tubules, filled with spermatic cells, radiated from the center of the testis (Figure 23 A, B). The mesorchium contained mesentery tissue that extended into the fold, and contained prominent blood vessels, nerve fascicles and loose aggregates of round pale-staining cells with central nuclei (Figure 23 B,C). The staining properties and shape of these cells within the mesentery resembled erythrocytes, seen within blood vessels (Figure 23 C, arrow). Three regions with putative Leydig cells were recognized in the testes of the round goby. 1) A vascularized aggregate of cells, characteristic of endocrine cells (polyhedral cells arranged in cords and clumps, with intertwining capillaries), lined one side of the fold adjacent to the mesorchial mesentery (Figure 23 B, D). 2) A second mass of putative Leydig cells was located adjacent to the spermatic (efferent) duct, located at the base of the fold (Figure 23 B, E). 3) Clusters of Leydig –like cells were present in the center of the testis (Figure 23 F). These were arranged into cords with capillaries coursing among

the cells (Figure 23 D, E, F). The toluidine blue staining intensity of the cytoplasm ranged from moderate (Figure 23 D) to dark (Figure 23 F). Examination of serial sections revealed that these Leydig-like cell aggregates formed "islands", rather than an uninterrupted arrangement along the length of the testis. Sperm were abundant within the spermatic (efferent) duct (Figure 23 E) and filled the lumina of seminiferous tubules (Figure 23 G). The 'pockets' of Leydig cells were not visible upon dissection, unlike the large single mass of glandular tissue in the black goby testis (Columbo and Burighel, 1974), thus making it infeasible to incubate the Leydig cells separately from the seminiferous tubules. Hence, whole testes (a mixture of glandular and spermatogenic tissues) were used for incubation studies.

3.3.2 Incubation of testes with [H^3]-androstenedione

Following HPLC separation (Figure 24), the incubation medium from fish I testes yielded seven prominent peaks of radioactivity (labeled A, C, D, D', E, F and G) and a region of indistinct, relatively minor peaks (labeled B). The incubation media from the testes of other four testes yielded a similar pattern of peaks. However, peak A was only present in one fish, and there was a new peak (labeled F') that was not present in fish I. Peak D' was evident only in fish I. The HCG-injected fish did not have an overtly different pattern from the non-HCG-injected fish (perhaps slightly higher amounts of F'). In all cases, Ad was > 95% converted to other steroids. In all cases also, > 90% of the radioactivity in each of the peaks (excluding region B) formed single major bands on TLC.

Identification studies were concentrated on steroids that were produced by testes from fish I. It proved possible through HPLC, TLC and microchemistry to match all the peaks to known C_{19} steroids (Table 4), except for D' and for one component of the B region (Table 5). The compound in the D' peak had an R_T value of 0.12. Although this value was close to that of three of the standards (Table 4), none of them co-migrated with the radioactive steroid after they had been mixed together and run on TLC. All the steroids that were identified had either an androsten-4-ene or a 5β-androstane configuration. None of the radioactive steroids co-migrated with any of the available 5α-androstane steroids. Microchemistry implemented in the identification of the incubation products is described in Table 6.

It was established that >90% of peak A and 50% of region B remained at the origin when run on TLC, by counting radio-activity at the origin . This made it likely that these steroids were conjugated to either a sulfate or a glucuronide group (making them too hydrophilic to chromatograph on TLC). In order to establish the nature of the conjugating group(s) in peak A, some of the radioactivity was run on an anion-exchange column with 10 μg each of the free, glucuronidated and sulfated forms of 17,20β-P. The bulk of the radioactivity eluted in a position (Figure 25) that was consistent with the steroid having a sulfate group (i.e. it was too strongly charged to be a glucuronide). This was confirmed by the fact that acid solvolysis turned > 90% of the radioactivity into a free steroid that was found to have the same mobility on TLC as peak E (11-oxo-ETIO). Acid solvolysis of fraction 37 (in region B) also released a steroid that had the same chromatographic properties as ETIO. Acid solvolysis would not have been expected to deconjugate glucuronide groups.

The identities of the main peaks from fish II testes were confirmed in the same way as for the first incubation (i.e. TLC plus microchemistry). Partial characterization (TLC with and without acetylation) was also carried on the steroid in peak F' to show that it was most probably testosterone. The identities of the steroids in the main peaks from testes in fish III, IV and V were only checked by co-migration on TLC.

Finally, the identities of solvolyzed peak A (11-oxo-ETIO), peak C (11-oxo-T), peak E (11-oxo-ETIO) and peak G (ETIO) were confirmed by recrystallization to constant specific activity (Table 7).

3.3.3 Incubation of a single testis (fish VI) with [H^3]-17-P

A single incubation was carried out with tritiated 17-P. HPLC separation of the incubation medium yielded at least nine peaks (Figure 26). Only a few of these peaks could be matched to the C_{19} steroids shown in Table 4. Also two of the peaks (*D* and *E*) yielded two prominent bands on TLC. Peak *A* had the same properties as peak A from testis I (i.e. it could be solvolyzed to a steroid that had the same mobility as 11-oxo-ETIO). Peak *F* also had the same mobility as 11-oxo-ETIO. The identities of these two steroids were confirmed by recrystallization to constant specific activity (Table 7).

Figure 23. The testis from a spermiating round goby. Semi-thin plastic section stained with toluidine blue.

A. A low power cross-sectional view of the testis. The black arrow points to a prominent fold located on the mesorchial side of the testis. Rectangles D, E and F show locations with glandular cells, shown in high power in panels D, E and F. The periphery of the testis (outlined with black squares) contains abundant seminiferous tubules filled with sperm. Rectangle G outlines a seminiferous tubule, shown at high power in panel G. A high power view of the mesorchial fold from a different specimen (outlined by a rectangle B) is shown in B.

B. The tissue surrounding the fold located on the mesorchial side of the testis. The arrow points to this fold. There are seminiferous tubules (st) located beside the fold, on one side, and a region with glandular cells on the opposite surface, enclosed by rectangle D, which is shown at high power in panel D. The tissue beneath the fold contains a spermatic duct (sd) and adjacent glandular cells, enclosed by rectangle E, enlarged in panel E. The region at the base of the fold, enclosed by an oval, contains structures shown at high power in panel C.

C. A high power view of mesentery, with an arteriole (a), nerve fascicle (nf) and polyhedral cells which appear to form part of the mesorchium, extending into the fold (arrow).

D. Glandular cells (putative Leydig cells, L) located beside the fold at the mesorchial surface (area 1 in B) shown at high power. Capillaries (c) with single erythrocytes are visible (arrows). The glandular cells are polyhedral, and arranged into cords and clumps. Seminferous tubules (st) are seen adjacent to the glandular cells.

E. Glandular cells (putative Leydig cells, L) located beneath the spermatic duct (sd), which is filled with sperm (sp). These round cells are arranged in a sizeable clump, with a single capillary (c) passing through this large cellular aggregate. The staining of these cells ranges from pale to moderate.

F. "Islands" of strongly basophilic putative Leydig cells (L) located in the center of the testis (location shown by a white arrow in A). In these cells, the cytoplasm is as strongly basophilic as the nucleus, and the nucleus is not distinguishable. Capillaries (c), containing erythrocytes, are prominent. Sperm (sp) within seminiferous tubules are beside these Leydig cell islands.

G. A high power view of a seminiferous tubule shows spermatic cells (sp) filling the lumen of the tubule. The small arrows outline the follicular cells of the seminiferous tubule.

Figure 23

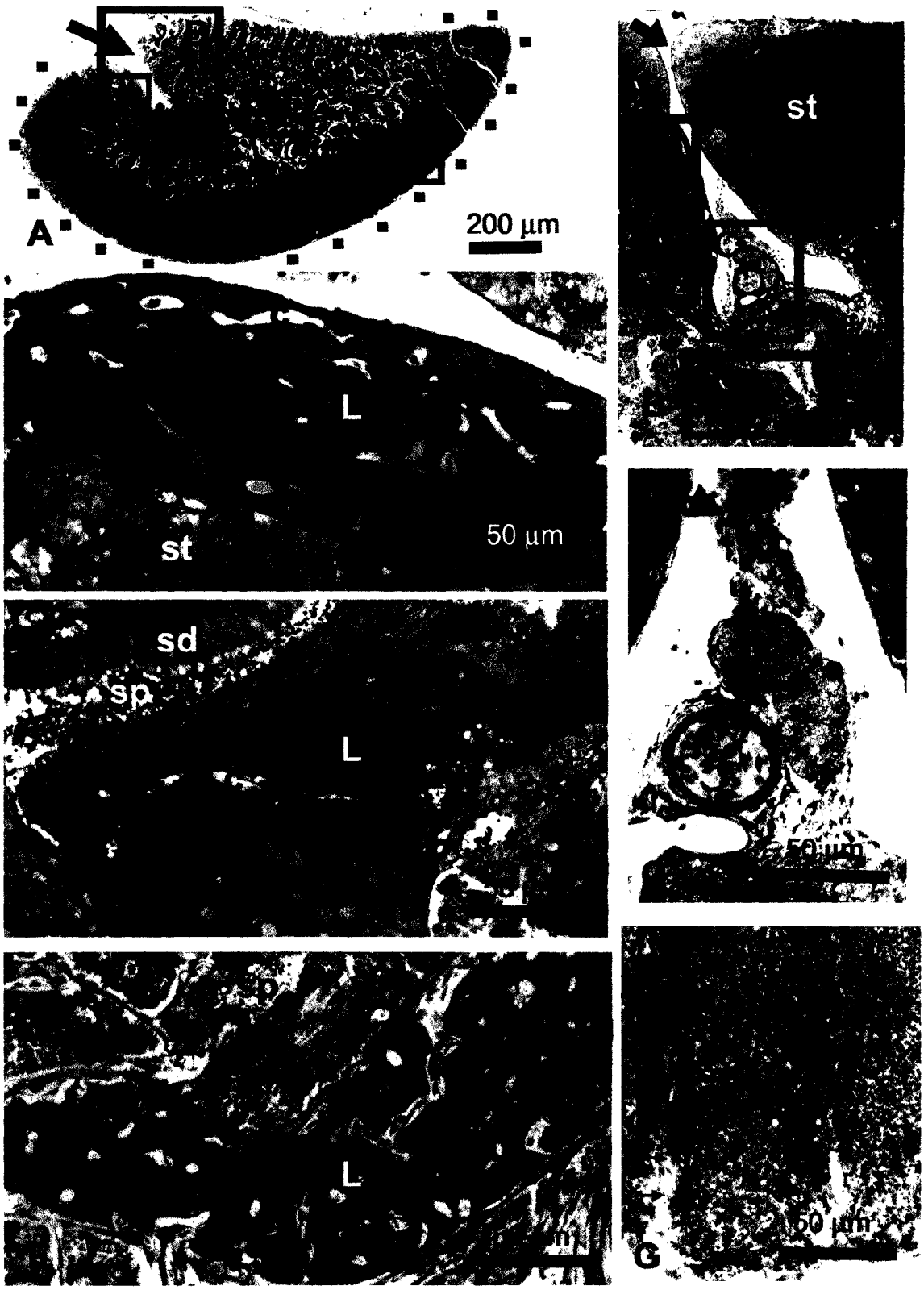
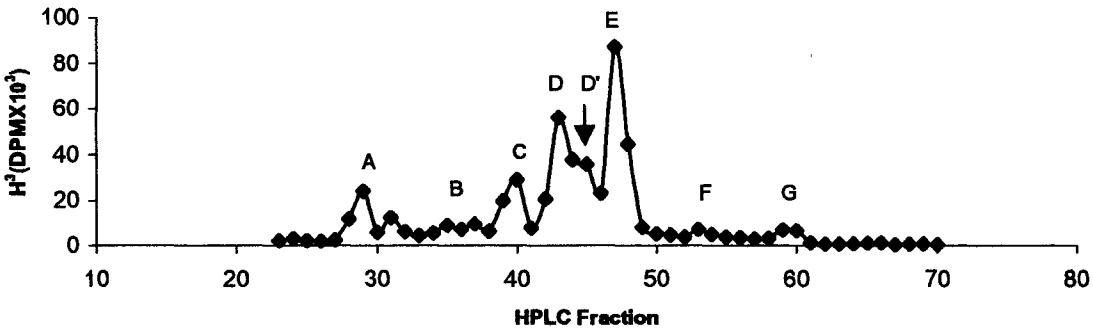
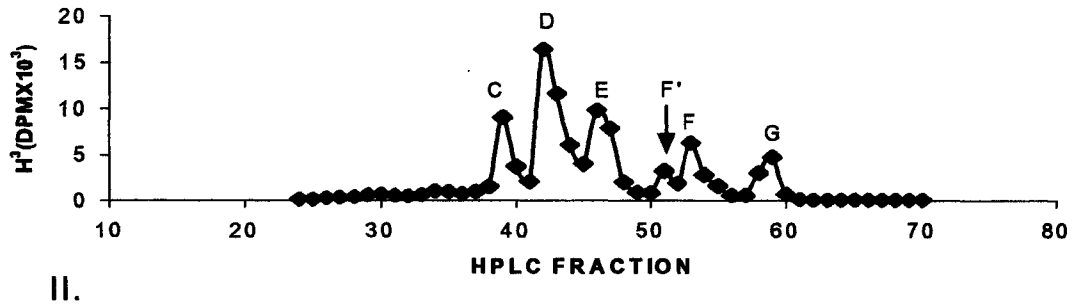


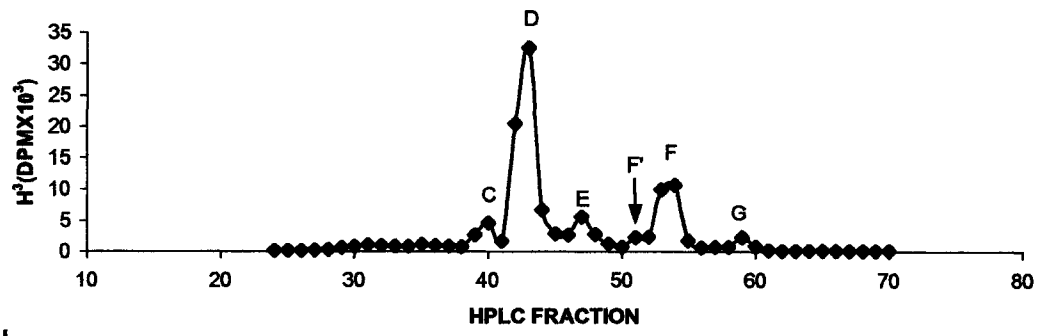
Figure 24. HPLC separation of media from round goby testes incubated with [^{3}H]-Ad. Each plot represents a single male (2 testes per plot). Fish II and III were injected with HCG the previous day. Decay per minute (dpm) is along the y axis and elution minutes along the x axis. See Table 2 for identities of the steroids that were found in each of the labeled peaks.



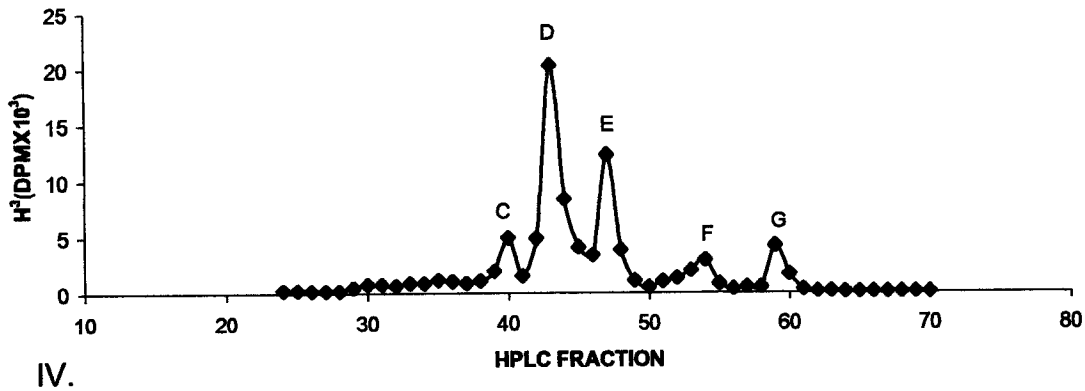
I.



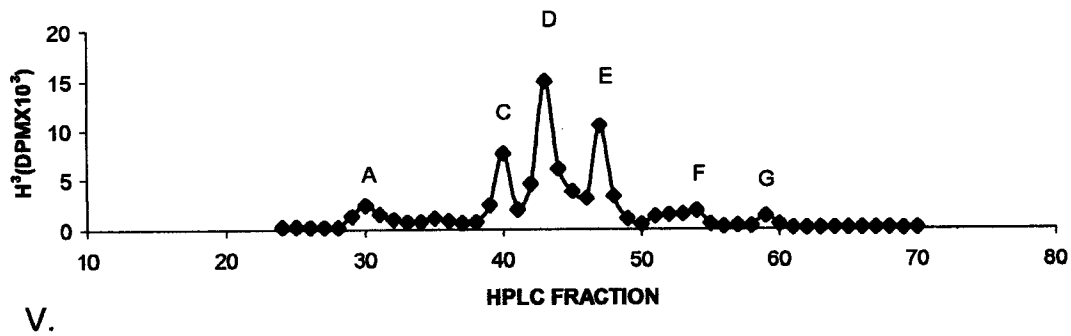
II.



III.



IV.



V.

Figure 25. DEAE-Sephadex (anion-exchange) separation of peak A from incubation of testes with [^{3}H]-Ad. The continuous curved line shows the elution position of radioactivity. The light grey curved line shows the UV absorption (at 254 nm) of free (F), glucuronidated (G) and sulfated (S) 17,20β-P. The continuous straight line shows the gradient. The elution position of the radioactivity is consistent with the radioactive compound being a sulfate.

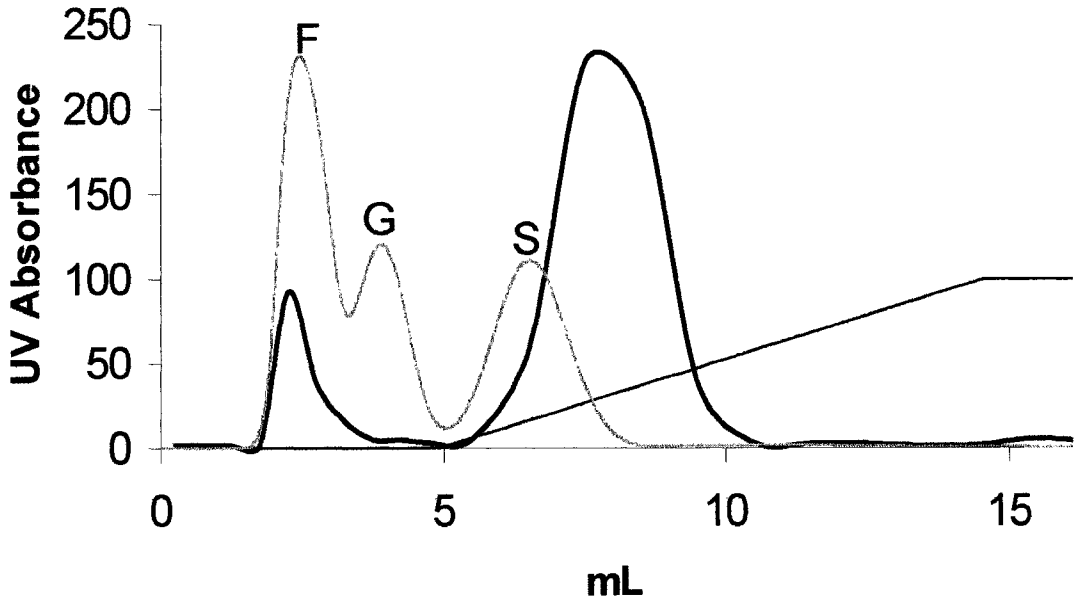
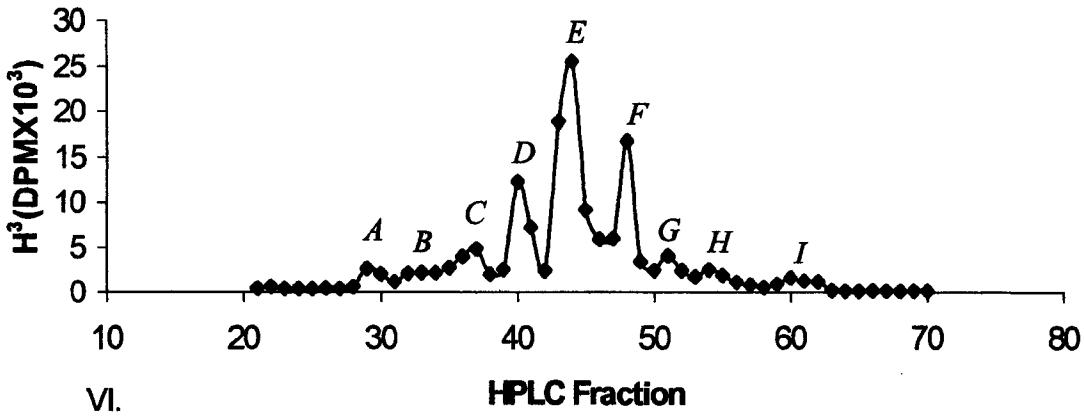


Figure 26. HPLC separation of media from testes of a single round goby male incubated with [^{3}H]-17-P. Tentative identifications were made of 11-oxo-T in peak *D* and Ad in peak *H*. Definitive identifications were made of 11-oxo-ETIO-s in peak *A* and 11-oxo-ETIO in peak *F*. The other steroids were not identified.



VI.

Table 5. Main steroids produced by incubation of testes of the round goby with [^{3}H]-Ad. Percent yields indicated in this table are expressed as a total of the radioactivity of all peaks made by testis I (Fig. 2)

PRODUCT	PEAK	% YIELD
11,17-dioxo-5β-androstan-3α-yl sulfate (11oxo-ETIO-s)	A	13.5
3α-hydroxy-17-oxo-5β-androstan-3α-yl sulfate (ETIO-s)	B	1.4 (1.7 unidentified)
17β-hydroxyandrost-4-ene-3,11-dione (11-oxo-T)	C	16.0
11β-hydroxyandrost-4-ene-3,17-dione (11β-OH-Ad)	D	18.3 (D' = 12.3% unidentified)
3α-hydroxy-5β-androstane-11,17-dione (11-oxo-ETIO)	E	28.5
androst-4-ene-3,17-dione (Ad)	F	3.9
3α-hydroxy-5β-androstan-17-one (ETIO)	G	4.33

Table 6. Identification evidence for peaks from testis 1. Microchemical reactions used were acetylation, oxidation, reduction and solvolysis. HPLC and TLC elution positions of each peak are also given.

Peak	HPLC (min)	R_T[a]	Identified Metabolite	Microchemistry Performed
A	28-30	origin	11,17-dioxo-5β-androstan-3α-yl sulfate (11-oxo-ETIO-s)	Following solvolysis: acetylation products comigrate on TLC; enzyme and chemical oxidation products have same mobility as 5β-androstane-3,11,17-trione on TLC.
B	37	origin	3α-hydroxy-17-oxo-5β-androstan-3α-yl sulfate (ETIO-s)	Following solvolysis: Acetylation products comigrate on TLC
C	39,40	0.67	17β-hydroxyandrost-4-ene-3,11-dione (11-oxo-T)	acetylation products comigrate on TLC; chemical oxidation product has same mobility as androst-4-ene-3,11,17-trione
D	43	0.66	11β-hydroxyandrost-4-ene-3,17-dione, (11β-OH-Ad)	failure to acetylate; enzyme reduction products comigrate on TLC; chemical oxidation product has same mobility as androst-4-ene-3,11,17-trione
D'	44	0.12	Unidentified	n/a
E	47	0.84	3α-hydroxy-5β-androstane-11,17-dione (11-oxo-ETIO)	acetylation products co-migrate on TLC enzyme and chemical oxidation products have same mobility as 5β-androstane-3,11,17-trione on both TLC and HPLC
F	53,54	1.70	Androst-4-ene-3,17-dione (Ad)	failure to acetylate
G	59,60	1.03	3α-hydroxy-5β-androstan-17-one (ETIO)	acetylation products comigrate on TLC enzyme oxidation product has same mobility as 5β-androstan-3,17-dione

A, due to day-to-day differences in TLC running conditions (e.g. temperature), these R_T values do not necessarily exactly match the values shown in Table 4.

Table 7. Results of recrystallization of several standard steroids with radioactive metabolites derived from incubation of round goby testes with either tritiated Ad or 17-P.

Cold standard	Radioactive steroid peak[a]:	Recrystallization number: 1	2	3	4
Testosterone (T)	E (original dpm=10750)[b]	1628[e]	176	74	69
Testosterone	tritiated T[c]	6337	6147	6206	5532
11-oxo-ETIO	E	4698	4421	4315	
11-oxo-ETIO	solv. A	9831	8985	8960	8383
11-oxo-ETIO	*F*[d]	9689	8733	8588	8763
11-oxo-ETIO	solv. *A*[d]	2401	1988	1850	1829
ETIO	G	2010	2153	1905	2331
11-oxo-T	C	5100	4576	4533	

a, letters refer to labeled peaks on HPLC (Fig. 24).
b, this was done as a negative control.
c, this was done as a positive control; tritiated testosterone was purchased from Amersham Pharmacia
d, these two steroids were derived from fish VI (17-P incubation; Fig. 26); solv. = solvolyzed
e, all values are in dpm/mg of crystal.

3.4 Discussion

Histological analysis showed that the testis of the round goby is similar to that of the black goby (Colombo and Burighel, 1974), of the rock goby *Gobius paganellus* (Stanley et al., 1965) and of the urohaze goby (Asahina et al., 1985), in possessing concentrations of Leydig cells. In these other species, the endocrine portion of the testis was found in a large glandular mass running along the mesorchium, as well as in smaller aggregates adjacent to the seminiferous tubule. The three spatially distinct zones with Leydig cells in the round goby match localizations previously observed in other gobiid species. The accumulation of Leydig cells adjacent to the fold facing the mesorchium in the round goby, parallels the large glandular mass (mesorchial gland) running along the mesorchium in the black goby (Colombo and Burighel, 1974) and the rock goby (Stanley et al., 1965) and in the central region of the urohaze goby testis (Asahina et al., 1985). Leydig cells concentrated beside the sperm duct have also been observed in the black goby, where the large mesorchial mass of Leydig cells extends as far as the collagenous sheath surrounding the deferent duct (Columbo and Burighel, 1974). The third location of Leydig cells in the round goby, scattered in pockets throughout the middle of the testes, is also an arrangement that has previously been observed in the rock goby (Stanley, 1965) and black goby (Colombo and Burighel, 1974; Rasotto and Mazzoldi, 2002). In the urohaze goby, cord-like processes with glandular cells extend between seminiferous tubules from the main central glandular mass (Asahina et al., 1985). The spatially distinct localization of Leydig cells in the testis of gobiids is different from the Leydig cell distribution in most vertebrate species, where the steroid producing endocrine Leydig cells are interstitial to the seminiferous compartment (e.g. Ross et al., 1989).

Circumstantial evidence that the glandular tissue in the testis is involved in the synthesis of pheromones in gobies comes from the work of Rasotto and Mazzoldi on the black goby (2002). These authors showed that there are two types of male – parents and 'sneakers'. Because the latter "sneak" fertilizations and do not guard or maintain nests, they do not presumably need to emit pheromones to attract females. Underlining this difference in behavior, the large glandular masses of Leydig cells are absent from the testes of the sneaker males. Furthermore, only the milt (ejaculate) of parental males has been shown to excite aggression in other parental males (Locatello et al., 2002).

The incubation of testes with [^{3}H]-Ad yielded steroids that either retained their androst-4-ene configuration or were 5β-reduced. Many were also 11-oxygenated. The presence of 5β-reductase, 11-hydroxylase and 11β-HSD enzymes in teleost testes is well-established (Borg, 1994; Fostier et al., 1983; Kime, 1993). Nevertheless, despite numerous reports of the synthesis of 11-oxo-T and ETIO in the testes of a variety of species, the synthesis of 11-oxo-ETIO does not appear to have been previously reported in any teleost, apart from a study on the tilapia *Sarotherodon mossambicus* (Kime and Hyder, 1983). However, in that study, the precursor steroid was already oxygenated at the 11-position (i.e. [^{3}H]-11-oxo-Ad), making it unclear whether tilapia would normally form it if the precursor had been Ad. In the present chapter, we have replicated this finding five times. We have also demonstrated the synthesis of this steroid from 17-P as well as from Ad.

There were only two other products from the 17-P incubation that we were able to match to any of the steroids in Table 4. These were 11-oxo-T and Ad (limited

identification only). The other peaks were not identified (through lack of resources). Some of them probably represent C_{21} metabolites.

In common with the black goby, the testes of the round goby produce an abundance of 5β-reduced steroids. Some of the steroids in the two species are the same (e.g. ETIO and ETIO-s). However, there are two major differences between the species. One difference is that the black goby seems to lack 11β-HSD activity. Although Colombo et al. (1977) found 11β-hydroxysteroids, they found no 11-oxo-steroids in the black goby incubations. Although this difference could be due to differences in protocol (different handling procedures, incubation media and precursors), it is unlikely. In the round goby, 11-oxo-ETIO and 11-oxo-T are not minor products. They form a large proportion of the metabolites in the incubation media – suggesting the presence of a lot of 11β-HSD activity. It is unlikely that Colombo et al. (1977) would have failed to find such activity in the black goby if it existed. The situation *in vivo* may be entirely different. There are several species in which it appears that it is common for C_{19} steroids to be secreted 'half-formed' from the testis and transformed to their final product by enzymes in either the blood cells (e.g. conversion of 11-oxo-Ad to 11-oxo-T in the stickleback *Gasterosteus aculeatus*; Mayer et al., 1990) or liver (e.g. conversion of 11β-OH-testosterone to 11-oxo-T in African catfish *Clarias gariepinus*; Cavaco et al., 1997).

The other difference between the round goby and the black goby is the apparent low amount of steroid conjugation in the round goby incubations. Although we found small amounts of the sulfated forms of ETIO and 11-oxo-ETIO, we were unable to conclusively demonstrate the presence of their glucuronidated forms (although they may have been present in trace amounts). In view of the results of Colombo et al. (1970;

1977), who found substantial production of both glucuronides and sulfates in both the rock goby and black goby, it seems surprising that we were unable to demonstrate the synthesis of ETIO-g. However, we caution against the interpretation that the necessary enzyme, steroid-UDP-glucuronosyl-transferase, is missing from the testes of the round goby. In one of their studies on the seminal vesicle of the African catfish, *Clarias gariepinus*, Schoonen and Lambert (1986) detected large amounts of steroid glucuronide formation. In a later study (Schoonen et al., 1987) they found hardly any at all. In this second study, they noted that there was a two hour delay between the capture of the animals and the incubation of their seminal vesicles and speculated that the capacity for producing glucuronides was lost more rapidly than the capacity for 5β-reduction and 11β-hydroxylation. In the present study, there was a relatively large (and unavoidable) 3 h delay between sacrifice of the animals and incubation of the testes. There was also evidence that steroid sulfotransferase activity might be labile; as 11-oxo-ETIO-s appeared to be low (or absent) in three of the incubations.

In several other species, temperature has been shown to influence glucuronide formation (Kime, 1980). However, the temperatures at which we and other authors have maintained the fish and subsequently carried out the incubations have been within the normal range for the species.

HCG injection was included within this study to determine whether it might radically alter the production of the steroids. However, although it only involved two fish, there was no clear indication that the HCG injection had any effect (perhaps a slight enhancement of T formation). It was therefore not investigated further.

Although there are similarities in steroid biosynthetic pathways between the black goby and the round goby, there appear to be very few between the urohaze-goby and the other two species. The urohaze-goby glandular tissue predominantly synthesized 5α-reduced steroids (Asahina et al., 1985). These were notably absent in the present study. However, in at least one species, the sailfin molly, *Poecilia latipinna*, Kime and Groves (1986) have demonstrated the production of both 5α- and 5β-reduced androgen within a single testis. Furthermore, both these types of androgen were present with either 3α- or 3β-hydroxyl groups.

One criticism that can be leveled at the present study is that incubations were carried out with whole testes, while Colombo et al. (1977) used just the mesorchial gland and Asahina et al. (1985) used separate 'glandular tissue' and 'seminiferous tissue'. I do not believe this is a problem. Firstly, apart from the fact that the structure of the testis in the round goby makes the clean dissection of glandular and seminiferous tissue infeasible, Asahina et al (1985) did in fact find no difference in the types of steroid produced by the testis of the urohaze goby - only in their yields.

One result for which there is no reasonable explanation is the presence of an unidentified metabolite (R_T=0.12; peak D') in the incubation medium from the testis of fish I, but its absence in testes from II through V. The relative mobility of the steroid suggests that it has at least two hydroxyl groups (of which there were several in Table 4 that we were unable to test). Since the incubation of testes from fish I was carried out in a different year and a different month from the others, the difference may be seasonal.

Conjugated steroids are more soluble in water than free steroids and therefore potentially more likely to be utilized as pheromones (Scott and Vermeirssen, 1994;

Vermeirssen and Scott, 1996). In mammals, conjugation is usually hepatic in origin and typically leads to excretion and deactivation of the steroid hormone (Kime, 1993). Colombo et al. (1979) showed not only that the testis of the male goby produced ETIO-g, but the steroid also induced behavioral activity when it was added to the water. Although we failed to establish the production of this particular steroid in the round goby testis, we have identified ETIO by itself and also, tentatively, ETIO-s, also identified in *Gobius jozo* by Colombo et al. (1977).

The probable route of excretion of free steroids is via the gills (Vermeirssen and Scott, 1996) and of sulfated and glucuronidated steroids via the urine and feces (Scott and Vermeirssen, 1994). The milt (Locatello et al., 2002) and seminal vesicle fluid (Schoonen and Lambert, 1986) are also potential sources of the pheromone. However, immunoassays first need to be developed for ETIO and 11K-ETIO in order to investigate these various possibilities.

More evidence that would point towards a pheromonal role for either 11-oxo-ETIO-s or ETIO-s is their elution positions on HPLC. Currently, water that has been conditioned by reproductive male round gobies has been separated by HPLC and each fraction has been tested against female round gobies for physiological activity (Bélanger et al., in press). The highest EOG activity came between fractions 30 to 40. This is a potentially significant finding, in that 11-oxo-ETIO-s, ETIO-s (and 11-oxo-ETIO-g) elute on HPLC between 30 and 40 min. None of these steroids was tested for EOG activity by Murphy et al. (2001). However, we have carried out preliminary studies on 11-oxo-ETIO, 11-oxo-ETIO-g and ETIO-s and found that they evoke an EOG response at least at 10^{-10}M (own unpublished data). However, 11-oxo-ETIO-s remains to be tested for EOG

activity and all four steroids remain to be tested for behavioral activity. Also, it still needs to be established that these steroids are actually produced, and released into the water, *in vivo* and that they also modify the behavior of the animals.

In conclusion, we have shown that the testis of the sexually mature male round goby contains islets of steroid-synthesizing glandular tissue. We have discovered that, *in vitro*, the testes produce at least four 5β-reduced C_{19} steroids (two of them novel). These findings lend further support to the hypotheses of Colombo et al. (1977) and Murphy et al. (2001) that sexually mature male gobies secrete pheromones that attract females and that these pheromones are derived from the testes and are probably steroids with a 5β-reduced configuration.

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CHAPTER 4: The in vitro biosynthesis of novel 5β reduced steroids in the seminal vesicle of the round goby, *Neogobius melanostomus*

4.1 Introduction

The parental male round goby (*Neogobius melanostomas*) is a benthic teleost fish that maintains and guards a nest into which many females deposit eggs (Wickett and Corkum, 1998; MacInnis and Corkum, 2000). It is likely that the male round goby releases a pheromone(s) to attract reproductive females to their nests, and that this pheromone(s) is a steroid originating in the gonads (Arbuckle et al., 2004; Murphy et al., 2001). Both 11-oxo-ETIO and 11-oxo-ETIO-s are two putative pheromones that are produced in the testis (Arbuckle et al., 2004). The seminal vesicle is a paired organ attached to the testes which are found in many species of the family Gobiidae (Moiseyeva et al., 1973; Fishelson, 1991; Lahnsteiner et al., 1992). They are lobular organs that have high secretory activity, which empty into the spermatic duct which leads to the genital papillae (Fishelson, 1991). Previous studies in fish have indicated that the seminal vesicle, an accessory male gonadal structure, also produces steroidal pheromones, as well as a mucoid fluid (Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988).

Strong evidence exists to support the hypothesis that the male round goby releases a pheromone (or a mixture of pheromones) that attracts reproductive females to their nests. In the black goby, *Gobius jozo* (=*G. niger*), from the family Gobiidae, males have been shown to emit a pheromone that attracts females to their nests (Mozzi, 1968). Colombo et al. (1974; 1977; 1980; 1982) have shown that the testes of black goby contains prominent Leydig (steroid-secreting) cells that produce predominantly conjugated and 5β-reduced steroids. The most prevalent of these was 17-oxo-5β-

androstan-3α-yl glucuronide (etiocholanolone glucuronide; ETIO-g). This steroid was also shown to act as an attractant to gravid females (Colombo et al., 1979; 1980). Furthermore, olfactory epithelial (electro-olfactogram, EOG) responses of the round goby indicated selective and strong responses to 5β reduced steroids (Murphy et al., 2001). As well in the round goby, EOG responses have indicated that reproductive male water is a strong olfactory stimulant in reproductive females, but not in non-reproductive females (Bélanger et al., 2004, in press). Taken together, research supports the view that the round goby releases steroids with pheromonal properties.

Previous incubations of the testis with [H^3]-androstenedione has indicated the production of two novel compounds, 11-oxo-etiocholanolone and 11-oxo-etiocholanolone sulfate (Arbuckle et al., 2004). These two steroids likely possess pheromonal activity in the round goby, given that the carbon A ring has a 5β-configuration that is linked with olfactory sensitivity (Murphy et al., 2001) and behavior induction (Colombo,1980) in two species of gobies. Furthermore, the production of a conjugated steroid (11-oxo-etiocholanolone sulfate) is significant, since conjugated steroids are more soluble in water than free steroids and therefore more likely to be utilized as pheromones (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996). In this study, the gonadal biosynthesis of steroidal compounds in the seminal vesicle of the round goby was examined in light of recent findings in the testis.

The main functions that have been attributed to the seminal vesicle are the temporary storage of sperm cells, the production of glycoproteins to facilitate fertilization and the production of reproductive pheromones (Nawar, 1960; Lahnsteiner et al., 1992; Schoonen and Lambert, 1986; Schoonen et al., 1987,1988). Although the first

description of the seminal vesicle was given by Eggert (1931), few studies have provided conclusive functional explanations for this accessory reproductive organ. The seminal vesicles are lined with excetory epithelium that contains columnar cells, excretory cells and interstitial, Leydig-type cells that may be part of the mesorchial gland (Fishelson et al., 1991). In the grass goby (*Zosterisessor ophiocephalus*), the seminal vesicle consists of secretory cells responsible for sialoglycoprotein secretions that are released together with spermatazoa to facilitate fertilization and adherence of eggs to sea grass (Lahnsteiner et al., 1992). The mucoid fluid produced by the seminal vesicle may function in plug formation after fertilization (von Ihering, 1937) and nutrition for the sperm cells (van Tienhoven, 1983). In the African catfish, Leydig type cells of the seminal vesicle are known to produce 5β reduced steroid glucuronides that function as sex pheromones (Schoonen and Lambert, 1986; Schoonen et al., 1987,1988); the secretions of the seminal vesicle may serve as a vehicle for excretion of this compound (Schoonen and Lambert, 1986b; Lambert et al., 1986; van Oordt, 1986; Resink et al., 1985). In the seminal vesicle of the grass goby, steroid producing cells are absent (Lahnsteiner et al., 1992). On the contrary, in the *Gobius paganellus* and *Gobius bucchichi*, groups of interstitial Leydig type cells are present between the tubules, embedded into the connective tissue of the boundary layers (Stanley et al., 1965). In the seminal vesicle in the round goby, interstitial tissue has been detected (Moiseyeva et al., 1973). Both the seminal vesicle and the testis develop from the embryonic genital ridge (van den Hurk and Resink, 1992). It is likely that the differentiation of the genital ridge into the testis and the seminal vesicle varies from species to species (Schoonen and

Lambert, 1986); this could account for the differing functions observed in the seminal vesicle in various teleosts.

Preliminary histology on the round goby testis and seminal vesicle indicates that the arrangement of the seminal vesicle with respect to the testis is parallel to that of other gobiids (Dr. S. Jasra, unpublished data). Each testis terminates into sperm ducts, where the seminal vesicles appear as paired organs (Fishelson, 1991). From here, the seminal vesicle and testis share a common genital pore which opens to the genital papillae (Lahnsteiner, 1992). Although a histological analysis has yet to be completed, a similar arrangement is likely in the round goby (Figure 27).

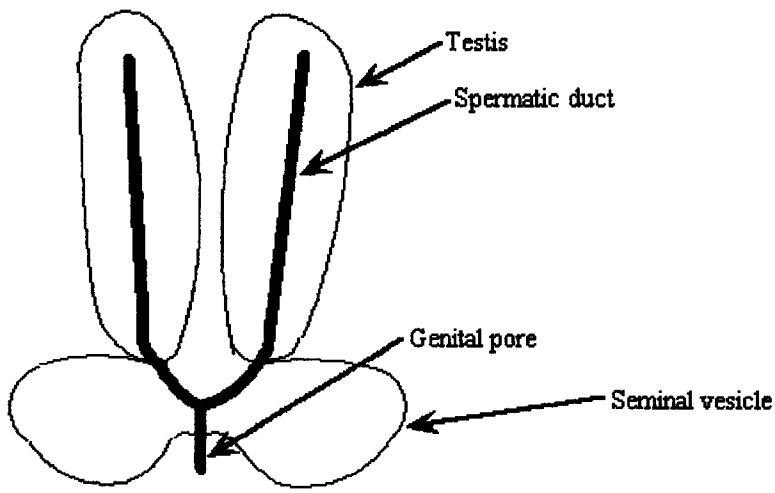


Figure 27. The arrangement of the male reproductive system in the family Gobiidae (adapted from Fishelson, 1991; Lahnsteiner, 1992 and personal observation).

The goal of this study was to determine if the seminal vesicle of the round goby possesses steroidogenic functions. This study addresses whether this accessory gonadal structure functions in conjunction with the testes in production and export of putative steroidal pheromone(s) into the aqueous environment. We determined the ability of the seminal vesicle to convert radioactive androstenedione (androst-4-ene-3, 20-dione; Ad) into other steroids *in vitro*. The identification studies were restricted to steroids formed

from tritiated Ad, since the relatively small number of other steroids into which Ad (a C_{19} steroid) can be converted, compared to other C_{21} precursors. The possibility exists that pertinent C_{21} steroids could be formed. However, previous studies have indicated that round gobies have specific and selectively strong responses to 5β reduced C_{19} steroids (Murphy et al., 2001); furthermore, work in the black goby indicated the abundant production of 5β-reduced C_{19} steroids which have pheromonal activity (Colombo et al., 1977; 1979; 1980; 1982).

The round goby is an invasive species of the Great Lakes and poses a threat to native fish species (Corkum et al., 1998). The identification of a male sex pheromone could possibly be implemented in a benign trap to attract females and disrupt spawning. A full understanding of the reproductive apparatus of the male round goby is required in order to accomplish this goal.

4.2 Materials and methods

4.2.1 Experimental animals

Round gobies were obtained from the Detroit River (Windsor, ON, Canada) by angling and were maintained at a water temperature of 18 to 20°C in dechlorinated tap water. Spermiating males were captured in June 2002 (sample I) or July 2003 (sample II). Spermiating males were identified by swollen cheeks and black coloration (MacInnis and Corkum, 2000). Furthermore, the presence of active sperm and histological examination of the testis was completed, confirming reproductive maturity. The fish were anaesthetized with MS222, killed, and the testes dissected and placed in ice-cold Leibowitz L15 medium (Sigma-Aldrich, USA). The seminal vesicles were identified as per Fishelson (1991) and Lahnsteiner et al. (1992).

4.2.3 In vitro incubation of the seminal vesicle with [^{3}H-Ad]

Four pairs of seminal vesicles were incubated with [1,2,6,7-^{3}H] androst-4-ene-3,17-dione; this radiochemical was purchased from Amersham Biosciences. The seminal vesicles were dissected from the fish, separated from the testis, finely minced, suspended in ice-cold Leibowitz L15 medium (Sigma) and transported on ice from the University of Windsor to Michigan State University (ca. 3 h). Here, the bits of tissue were distributed to fresh 50 ml plastic tubes containing 10 ml Leibowitz L15 medium and 10 to 50 μCi tritiated Ad. The tubes were laid on their side and gently agitated at 16 °C for a further 6 h (Kime and Scott, 1993). At the end of the incubation period, the medium was filtered and then passed through a Sep-Pak C-18 cartridge (Waters Chromatography, Millipore,

Milford, MA, USA). This was washed with 5 ml distilled water and then eluted with 5 ml methanol. The extract was stored at –20 °C.

4.2.4 Separation of tritiated metabolites from reproductive male round goby by High-Performance Liquid Chromatography (HPLC)

The methanol extracts from the Sep-Pak C-18 cartridges were dried down either under a stream of nitrogen at 45°C or in a rotary evaporator, mixed with 10 μg each of Ad and 11-oxo-T, reconstituted in 1 ml acetonitrile/water/trifluoroacetic acid (28/72/0.01; v/v/v) and then loaded onto an analytical reverse-phase HPLC column (Rainin Dynamax Microsorb; 5μm octadecylsilane; 4.6mm x 25 cm; fitted with a 1.5 cm guard module). Two pumps were used to deliver solvents through the column at a rate of 0.5 ml/min. Solvent A was 0.01% trifluoroacetic acid (TFA) in distilled water and solvent B was 70% acetonitrile and 0.01% TFA in distilled water: 0-10 min, 28.6% B; 10-60 min, 28.6-100% B; 60-80 min, 100% B; 80-82 min, 100-28.6% B; 82-100 min, 28.6% B. One minute fractions were collected between 20 and 70 minutes. A volume of 5μl from each HPLC fraction was then counted in a scintillation counter and fractions that formed distinct peaks on HPLC were saved for identification studies. Part of the identification criteria for metabolites involved the position that they eluted on HPLC. This was established for the synthetic standards by running 10 or 20 μg of each on the HPLC column under the same running conditions as above and monitoring the eluate with a diode-array detector. Steroids with a 4-ene configuration (e.g. Ad and 11-oxo-T) gave a strong peak of UV adsorption at ca. 248 nm. Steroids with a C=O group (or groups) gave a much weaker, but nevertheless distinct, peak of UV adsorption at ca. 200 nm.

4.2.5 Identification of steroids: Thin-Layer Chromatography (TLC) of HPLC Fractions

The standard synthetic steroids used in this study (Table 8) were purchased from Sigma Chemical Company or Steraloids. We tried to obtain all the steroids into which Ad might be converted via 'well-established' biosynthetic pathways. Using this criterion, we might expect there to be metabolites with either androst-4-ene, 5α-androstan or 5β-androstan configurations. In the 5α- and 5β-reduced steroids, we might expect to find either a 3-oxo, a 3α-hydroxyl or a 3β-hydroxyl group. In the androst-4-ene steroids, we would expect to find only a 3-oxo group. We would not expect to find 3-reduced androst-4-ene steroids as, to our knowledge, direct reduction of the 3-oxo group of 4-ene steroids has not been demonstrated in any vertebrate (although such steroids may be formed when 5-ene steroids are used as intermediates; Inaba et al., 1966). We might expect the 17-oxo group to be either retained or reduced to form a 17β-hydroxyl (but not 17α-hydroxyl) group. We might expect the 11-carbon to remain as it is or to be 11-oxygenated to form an 11β-hydroxyl (but not 11α-hydroxyl) group and then also further oxidized to form an 11-oxo group. All these possible combinations are shown in Table 8 (including eight steroids that we were unable to obtain).

There is always the possibility that Ad will be converted to C_{18} (estrogen-like) steroids. However, we have not included these in Table 8 as estrogen formation by testes has not been reported in teleosts (Fostier et al., 1983). No estrogen formation was found in previous studies on the testes of black goby (Colombo et al., 1977) or urohaze-goby *Glossogobius olivaceus* (Asahina et al., 1985).

We might also expect that any steroid in Table 8 that has either a 3α-oxo, 3β-oxo or 17β-oxo group could be glucuronidated or sulfated. Identification of conjugated steroids normally involves their deconjugation through either enzyme hydrolysis (which works on glucuronides and some sulfates) or acid solvolysis (which works on sulfates only) (Scott and Vermeirssen, 1994). Since neither glucuronidated or sulfated steroids were identified in this study, neither of these techniques were employed.

All steroids were dissolved in ethanol at a concentration of 500 μg/ml. At the outset of the identification work, 10 μg of each steroid was combined with 10 μg testosterone (T), spotted onto separate lanes of the TLC plates and developed with chloroform/methanol (50/2, v/v). The positions of the androst-4-ene steroids (e.g. Ad, T, 11-oxo-T) were detected by shining a UV lamp at the plate. Steroids with reactive hydroxyl groups (e.g. 3α-hydroxy-5β-androstane-11,17-dione; 11-oxo-ETIO) were detected by spraying with a solution of phosphomolybic acid in ethanol (10% w/v) and heating the plate for 5 min at 150°C. Steroids with no reactive hydroxyl groups (e.g. 5β-androstane-3,11,17-trione) were detected by spraying with concentrated sulfuric acid in methanol (10% v/v) and heating the plate for 5 min at 150°C. After the bands were revealed, their distance to the origin was measured to the nearest millimeter. The relative position of each steroid in relation to that of T was then calculated. This ratio (R_T) formed the basis of a table (Table 8) against which the relative elution positions of radioactive bands could subsequently be compared.

To determine the identity of the radioactive steroids in the various peaks, ca. 20,000 dpm was mixed with 10 μg T, dried down, reconstituted in 30 μl ethyl acetate and spotted and run on TLC as described above. The position of T was marked with a pencil

and each lane then divided into 5 mm strips that were scraped off, mixed directly with 7 ml scintillation fluid and counted. The position of the radioactivity relative to that of T was then compared with the R_T values in Table 8. By doing this, it was possible to narrow down the range of possible matching standards for each HPLC peak. A more precise match was obtained by mixing 20,000 dpm of radioactive steroid with 10 μg each of those steroids that had the most similar R_T values and then running them together on TLC. After the bands had been revealed, they were marked and scraped off, as was 2 x 0.5 cm of silica gel from either side of the bands. All the scrapes were mixed with scintillation fluid and counted. In any situation where the radioactivity was > 90% associated with a band, microchemical studies were then carried out to further establish the co-identity of radioactive metabolite and standard steroid.

Table 8: Standard synthetic steroids against which the tritiated metabolites were compared. R_T is the relative position of each steroid in relation to testosterone (T) when run on TLC. HPLC elution times were only determined for steroids of interest.

Steroid[a]	Common name	R_T[b]	HPLC elution (minutes)
Available steroids:			
3α,17β-dihydroxy-5β-androstan-11-one		0.12	
5α-androstane-3β,11β,17β-triol		0.14	
11β,17β-dihydroxyandrost-4-en-3-one	11β-OH testosterone	0.16	40
3β,11β-dihydroxy-5αandrostan-11-one		0.27	
17β-hydroxyandrost-4-ene-3,11-dione	11-oxo-testosterone (11-oxo-T)[c]	0.4	40
3β,11β-dihydroxy-5α-androstan-17one		0.44	
3α,11β-dihydroxy-5β-androstan-17-one		0.45	
5β-androstane-3α,17β-diol		0.48	
17β-hydroxy-5β-androstane-3,11-dione		0.48	45
3α,11β-dihydroxy-5α-androstan-17-one		0.53	
3β,11β-dihydroxy-5β-androstan-17-one		0.57	
17β-hydroxy-5α-androstane-3,11-dione		0.58	
3α-hydroxy-5β-androstane-11,17-dione	11-oxo-etiocholanolone (11-oxo-ETIO)	0.67	47
11β-hydroxyandrost-4-ene-3,17-dione	11β-OH androstenedione (11β-OH-Ad)	0.72	43
5α-androstane-3α,17β-diol		0.72	
3α-hydroxy-5α-androstane-11,17-dione		0.77	
5α-androstane-3β,17β-diol		0.78	
3β-hydroxy-5α-androstane-11,17-dione		0.79	
5β-androstane-3β,17β-diol		0.86	
17β-hydroxyandrost-4-ene-3-one	Testosterone (T)	1.0	52
3α-hydroxy-5β-androstan-17-one	Etiocholanolone (ETIO)	1.0	60
11β-hydroxy-5α-androstane-3,17-dione		1.03	
11β-hydroxy-5β-androstane-3,17-dione		1.06	
17β-hydroxy-5β-androstan-3-one		1.12	
3β-hydroxy-5α-androstan-17-one		1.14	
3α-hydroxy-5α-androstan-17-one		1.20	
17β-hydroxy-5α-androstan-3-one		1.28	
3β-hydroxy-5β-androstan-17-one		1.28	
5β-androstane-3,11,17-trione		1.33	48
androst-4-ene-3,11,17-trione	11-oxo-androstenedione; androstenetrione	1.38	45
5α-androstane-3,11,17-trione		1.49	47
androst-4-ene-3,17-dione	Androstenedione (Ad)	1.70	55
5β-androstane-3,17-dione		1.82	

5α-androstane-3,17-dione		1.94	
Conjugated steroids:			
17-oxo-5β-androstan-3α-yl glucuronide	Etiocholanolone glucuronide (ETIOg)	0	44
17-oxo-5β-androstan-3α-yl sulfate	Etiocholanolone sulfate (ETIOs)	0	37
11,17-dioxo-5β-androstan-3α-yl glucuronide	11-oxo-etiocholanolone glucuronide (11-oxo-ETIOg)	0	38
11,17-dioxo-5β-androstan-3α-yl sulfate	11-oxo-etiocholanolone sulfate (11-oxo-ETIOs)	0	30
Steroids (in same range as above) that were not available for testing:			
5α-androstane-3α,11β,17β-triol			
5β-androstane-3β,11β,17β-triol			
5β-androstane-3α,11β,17β-triol			
3α,17β-dihydroxy-5α-androstan-3-one			
11β,17β-dihydroxy-5α-androstan-3-one			
3β,17β-dihydroxy-5β-androstan-11-one			
11β,17β-dihydroxy-5β-androstan-3-one			
3β-hydroxy-5β-androstane-11,17-dione			

[a] the nomenclature of steroids in this chapter follows that recommended by IUPAC (Kime, 1995; www.chem.qmul.ac.uk/iupac/steroid) and not that used by Steraloids Inc. (www.steraloids.com), Sigma-Aldrich (www.sigma-aldrich.com) or Murphy et al. (2001).

[b] the R_T values and HPLC elution times shown in Table 8 are only approximate and are expected (and were found) to differ slightly in response to temperature and exact composition of solvents; however, approximate R_T provided a useful 'first step' in the identification of products from incubation of testis with tritiated Ad.

[c] also known incorrectly as 11-keto-testosterone

4.2.6 Microchemical reactions

Prior to each microchemical reaction, the radioactive metabolite (ca. 20,000 dpm) and the cold standard (10 to 20 μg) were mixed together and evaporated under a stream of nitrogen at 45°C. In some situations, in addition to the above, the radioactive steroid was treated in the absence of cold standard and then mixed with 10 μg of the 'expected' product prior to being run on TLC.

Enzymic oxidation: The steroid was redissolved in 1 ml 0.05M Tris-HCl (pH 9) containing 1.5 mg NAD (Sigma N-1511) and 1.2 mg Hydroxysteroid Dehydrogenase (HSD; Sigma H-8879). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate.

Enzymic reduction: The steroid was redissolved in 1 ml 0.05M Tris-HCl (pH 7.6) containing 3.3 mg HSD (Sigma, H8879) and 2.4 mg NADH (Sigma, N-8179). The reaction mixture was left overnight at room temperature and then extracted twice with 4 ml ethyl acetate.

Acetylation: The steroid was redissolved in 100 μl pyridine and 100 μl acetic anhydride and left overnight. The pyridine and acetic anhydride were removed by drying down with a stream of nitrogen at 45°C. The plates were developed with chloroform/ethanol 50/1 (v/v) to prevent potential acetylated products from migrating too far up the plate.

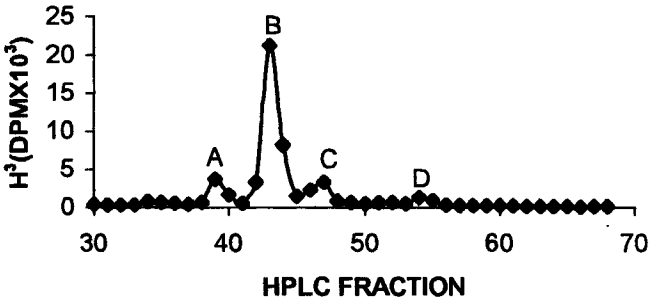
4.3 Results

4.3.1 Incubation of seminal vesicle with [H^3]-androstenedione

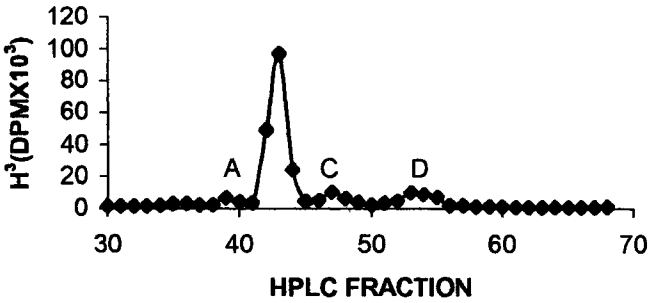
Following HPLC separation (Figure 28), the incubation medium from samples I and II yielded four prominent peaks of radioactivity (labeled A, B, C, and D). In both incubations, Ad was >95% converted into other steroids. In order of abundance, the following steroids were produced from the conversion of [H^3]-Ad: 11-oxo-androstenedione, 11-oxo-testosterone, and 11-oxo-etiocholanolone. All of the steroid metabolites identified had either a 5β-androstane or androsten-4-ene configuration. Furthermore, all steroids produced were 11-oxygenated. This would indicate the presence of an abundance of 11β-HSD in the seminal vesicle.

The metabolites produced by the seminal vesicles of samples I and II were determined by implementing HPLC, TLC and microchemistry. It was possible to match all of the peaks to the known C_{19} steroids (Table 9). Microchemistry implemented in this study consisted of acetylation, enzymatic oxidation or enymatic reduction (Table 10). In the incubation of the seminal vesicle, no conjugated steroids were detected.

Figure 28. HPLC separation of media from round goby seminal vesicle incubated with [^{3}H]-Ad. Each plot represents two pairs of seminal vesicles (four individual seminal vesicles) from two reproductive males. Sample I is from two fish in the summer of 2002. Sample II is from two fish from the summer of 2003. Decay per minute (dpm) is along the y axis and elution minutes along the x axis. See Table 19 for identities of the steroids that were found in each of the labeled peaks.



I.



II.

Table 9. Main steroids produced by incubation of the seminal vesicle of the round goby with [H³]-Ad. Percent yields indicated in this table are expressed as a total of the radioactivity of all peaks of seminal vesicle I (Figure 27).

PRODUCT	PEAK	% YIELD
4-Androsten-17β-ol-3,11-dione (11-oxo-testosterone)	A	12.3
4-Androsten-3,11,17-trione (11-oxo-androstenedione)	B	78.4
5β-Androstan-3α-ol-11,17-dione (11-oxo-etiocholanolone)	C	5.3
4-Androsten-3,17-dione (Androstenedione)	D	4.1

Table 10. Microchemistry implemented in the identification of incubation products from the seminal vesicle of the round goby. Microchemical reactions used were acetylation, oxidation, and reduction. Microchemistry performed to confirm identification is outlined. HPLC and TLC elution positions of each peak are given.

Peak	HPLC (min)	R_T[a]	Identified Metabolite	Microchemistry Performed
A	39,40,41	0.56	4-Androsten-17β-ol-3,11-dione (11-oxo-testosterone)	Acetylation products comigrate on TLC
B	42,43,44	1.23	4-Androsten-3,11,17-trione (11-oxo-androstenedione)	Neither standard or radioactive metabolite acetylate on TLC Enzymatic reduction product has the same mobility as 4-androstene-17β-ol, 3,11-dione on TLC
C	47	0.74	5β-Androstan-3α-ol-11,17-dione (11-oxo-etiocholanolone)	Enzymatic oxidation has the same mobility as 5β-androstan-3,11,17 trione on TLC
D	54	1.38	4-Androsten-3,17-dione (Androstenedione)	Enzymatic reduction has the same mobility as 4-androsten-17β-ol-3-one

A, due to day-to-day differences in TLC running conditions (eg. Temperature), these R_T values do not necessarily exactly match the values shown in Table 8.

4.4 Discussion

The incubation of the seminal vesicle with [^{3}H]-Ad yielded steroids that either retained their androst-4-ene configuration or were 5β-reduced. All steroid metabolites produced were 11-oxygenated. The presence of 5β-reductase, 11-hydroxylase and 11β-HSD enzymes in teleost testes (Borg, 1994; Fostier et al., 1983; Kime, 1993) and seminal vesicle has been established (Schoonen et al., 1987). Besides a study in the testes of the tilapia *Sarotherodon mossambicus* (Kime and Hyder, 1983), it seems that the synthesis of 11-oxo ETIO has not been indicated in any teleost. Furthermore, in the *Sarotherodon mossambicus*, the precursor steroid was already oxygenated at the 11-position (i.e. [^{3}H]-11-oxo-Ad); whether the tilapia would form 11-oxo-ETIO from a non-oxygenated compound such as Ad was not determined. We found this compound during two different reproductive seasons (summers of 2002 and 2003) in the seminal vesicle. Furthermore, this compound was also identified in the testis from the conversion of both [^{3}H]-Ad and [^{3}H]-17-P (Arbuckle, et al., submitted, 2004) during the summer of 2002 and 2003.

Since both the seminal vesicle and the testis develop from the embryonic genital ridge (van den Hurk and Resink, 1992), it is likely that the differentiation of the genital ridge into the testis and seminal vesicle varies from species to species (Schoonen and Lambert, 1986). This could account for the fact that the division of functions between the seminal vesicle and the testis tends to be slightly different in various species (African catfish, Schoonen and Lambert, 1986b; Schoonen et al., 1987, 1988; zebrafish, van den Hurk, et al., 1987c). In the African catfish, the seminal vesicle is known to synthesize a larger amount of steroid glucuronides and a greater variety of them, than the testes; these compounds have known pheromonal functions (Schoonen and Lambert, 1986b;

Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988). In fish without seminal vesicles, the steroid hormones and the steroid glucuronides are synthesized solely in the testis (eg. zebrafish; van den Hurk, et al., 1987c). In the *Gobius jozo* and *Gobius paganellus*, which have well developed seminal vesicles, the synthesis of steroid conjugates (glucuronides) was reported only in the testes (Colombo et al., 1970, 1977). Hence, the *Gobius jozo* and *Gobius paganellus* can be paralleled to the round goby. We have shown in a previous study (Arbuckle et al., 2004), that steroid conjugates are produced in the testes. In this study in the seminal vesicle, no conjugated steroids were detected. In a previous study on the testis of the round goby, clusters of Leydig cells produced 11-oxo-etiocholanolone-sulfate, which is a putative pheromone (Arbuckle, et al., submitted, 2004). Hence, there is a varied division of function with respect to steroid biosynthesis in the testes and seminal vesicle of teleosts.

Many functions have been attributed to the seminal vesicle. This includes the temporary storage of sperm cells (Nawar, 1960) and the production of a glycoprotein secretions that are released with the sperm to facilitate fertilization and adherence of eggs to sea grass (Lahnsteiner et al. 1992). In males of the family Gobiidae, these glycoproteins have been shown to mix with sperm, whereby males produce sperm trails in the nest before and after egg deposition (Marconato et al., 1996). In the African catfish, the fluid produced by the seminal vesicles has been suggested to be a vehicle for steroid glucuronides to be excreted and act as pheromones (Schoonen and Lambert, 1986b; Lambert et al., 1986; von Oordt, 1986; Resink et al., 1985). Furthermore, in the African catish, the seminal vesicles have been implicated in the production of

pheromones (Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988). These are all possible functions of the round goby seminal vesicle.

The seminal vesicle and testis share a common genital pore which opens to the genital papillae (Lahnsteiner, 1992). In the *Buenia jeffreysii* and *Pterogobius virgo* the testis and seminal vesicle tissue mingle at their point of junction with the sperm duct (Fishelson, 1991). In the *Gobius niger*, the transition from the testicular to seminal vesicle tissue along the sperm duct is gradual. A similar arrangement is highly likely in the round goby. Hence, the free 11-oxo-ETIO produced in the seminal vesicle could possibly become conjugated along its path towards the genital pore, leading to the aqueous environment. The epithelium of the sperm duct could possibly conjugate 11-oxo-ETIO. As well, the point of junction at the sperm duct where the testis and seminal vesicle tissue "mingle" could conjugate 11-oxo-ETIO. A concentration of Leydig cells around the sperm duct (the posterior end of the testis, which attaches to the seminal vesicle), has been shown in the round goby (Arbuckle et al., 2004). This area that composes testis and seminal vesicle at their point of junction is likely to be dense with steroid sulfotransferase, as testicular tissue near this junction has been suggested to produce 11-oxo-ETIO-sulfate (Arbuckle et al, 2004). Hence, this is a probable site of conjugation of the free 11-oxo-ETIO produced in the seminal vesicle. The other two steroids produced in the seminal vesicle, 11-oxo-testosterone and 11-oxo-androstenedione could also contribute to the pheromonal mileu putatively secreted from the male round goby. 11-oxo-testosterone has known hormonal functions (Kime, 1993) and is responsible for stimulating spermatogenesis and male secondary characteristics (Miura et al., 1991).

The production of conjugated steroids in fish is significant, since they are more water soluble than free steroids and therefore are potentially more likely to be utilized as pheromones (Scott and Vermeirssen, 1994; Vermeirssen and Scott, 1996). The likely route of excretion of free steroids is via the gills (Vermeirssen and Scott, 1996) and of sulfated and glucuronidated steroids is via the urine and feces (Scott and Vermeirssen, 1994). In mammals, enzymes for the conjugation of steroids are typically located in the liver (Colombo et al., 1980; Kime, 1993); fish are unique in that many of these enzymes for conjugation are located within the gonad itself (Kime, 1993). Conjugated steroids have been shown to be produced in the gonads of the round goby, black goby and the African catfish (Colombo et al., 1974, 1977, 1980, 1982; Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988; Arbuckle et al., 2004). Furthermore, conjugated steroids produced in the gonads of the African catfish and the black goby possess pheromonal properties (Colombo et al., 1980; Schoonen and Lambert, 1986; Schoonen et al., 1987, 1988). It appears that in teleost fish, the gonads are specialized in producing conjugated pheromonal steroids; hence in the round goby, the conjugation of steroids hepatically, although possible, is highly unlikely. Immunoassays need to be developed in order to investigate the various possibilities proposed here. Furthermore, immunocytochemical and histochemical studies co-localizing key steroid enzymes (especially steroid sulfotransferase) with respect to the seminal vesicle, testis and the spermatic duct is required in order to validate these possibilities.

This study has shown that the seminal vesicle is steroidogenic, in that it converts [^{3}H]-Ad into other steroids. *In-vitro*, the seminal vesicle produced steroids that were either androst-4-ene configuration or were 5β-reduced C_{19} steroids. All three steroids

produced were 11-oxygenated. One of these steroids, 11-oxo-etiocholanolone, was in fact novel. Further studies are required to confirm the possible pheromonal functions of these compounds produced *in-vitro*, on the premise that the 5β reduced steroid configuration elicits strong physiological responses in females (Murphy et al., 2001).

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CHAPTER 5: General Conclusions

The objective of this thesis was to study the production and olfactory detection of pheromones in the round goby and the sea lamprey. Both are invasive species in the Great Lakes (Corkum et al., 1998; Li et al., 2002); furthermore, pheromone communication seems to fundamental their reproductive success and population expansion (Li et al., 2002; Murphy et al., 2001). The separate studies presented here provide insight to the understanding of pheromone communication in vertebrates; this knowledge could possibly lead to an environmentally benign method of species control.

In chapter 2, the spatial organization of the olfactory system in the sea lamprey was studied using neuronal tract tracers. This study indicated that medial, non-G_{olf}-IR glomeruli projects topographically to a specific ventral region of the olfactory epithelium. Hence, a medial-ventral pathway in the larval sea lamprey seems to exist, as defined by functional and spatial parameters.

This finding is significant for many reasons. Firstly, in most fish species, topographic projections between the olfactory bulb and olfactory epithelium is absent (Hara and Zhang, 1996). Secondly, the projection of a specifc glomerular subset with unique G-protein expression (i.e. G_{olf} negative) to a specific region of the peripheral olfactory organ has never been shown before in the sea lamprey. Furthermore, these findings are significant because the medial olfactory pathway and tract has been implicated in the detection of pheromones in many fish species (Hara and Zhang, 1996; Friedrich and Korsching, 1998; Weltzien et al., 2003). The medial olfactory tract in fish may be functionally similar to the VNO in the olfactory system of mammals (Eisthen, 1992; Dulka, 1993). Taken together, this study indicates that the differential spatial

expression of alternate G proteins within the primary olfactory pathway in vertebrates may have evolved before gnathostome radiation or in parallel.

In Chapters 3 and 4, the production of putative reproductive pheromones in the round goby was studied. The round goby is an invasive species of the Great Lakes and poses a threat to native fish species (Corkum et al., 1998). The identification of a male sex pheromone could possibly be implemented in a benign trap to attract females and/or disrupt spawning. A full understanding of the reproductive apparatus of the male round goby is essential to garner insight into pheromonal communication. Both the testis and seminal vesicle were incubated with tritiated precursors to determine the *in vivo* production of steroids in this species. Results indicated the production of primarily 5β-reduced C_{19} steroids, which included two novel compounds, 11-oxo-ETIO and 11-oxo-ETIO-s. The production of 11-oxo-ETIO-s was indicated only in the testis; the free form of this compound was present in both reproductive structures. These two compounds are likely pheromonal candidates given that the carbon A ring has a 5β-configuration, that has been linked with olfactory sensivity and behavior induction in two species of gobies (Murphy et al., 2001; Colombo et al., 1980). The *in vivo* production of these compounds and pheromonal activity remains to be established. In summary, this study indicates the production of putative steroidal pheromones in the male round goby which may be useful for trapping females and/or disrupting their spawning activities.

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Appendix 1: Recipes

Stock A = 27.6g sodium phosphate monobasic (NaH_2PO_4) in 1L distilled water

Stock B = 53.7g sodium phosphate dibasic (Na_2HPO_4) in 1L of distilled water

0.2M Phosphate Buffer (PB), pH=7.4
190 mL of stock A
810 mL of stock B

0.1M Phosphate Buffer, pH=7.4
1L of 0.2M Phosphate buffer
1L of distilled water

0.1M Phosphate Buffered Saline (PBS), pH=7.4
8g Sodium Chloride (NaCl)
0.2g Potassium Chloride (KCl)
1L of 0.1M Phosphate buffer

1° Antibody Diluent for GS-1 isolectin B_4, pH=7.5
10mM HEPES
0.15M NaCl
0.2% Triton-X

2° Antibody Diluent for GS-1 isolectin B_4, pH=7.5 (Texas Red avidin-fluorescein DCS)
1:100 antibody dilution, composed of 10µl of 2° Antibody added to 990µl dilution buffer (0.1M sodium bicarbonate, 0.15M NaCl)

Sucrose solutions
10% sucrose: 10 g sucrose in 100 mL of 0.1M PB
20% sucrose: 20 g sucrose in 100 mL 0.1M PB
30% sucrose: 30 g sucrose in 100 mL 0.1MPB

4% Paraformaldehyde
4 g paraformaldehyde
50 mL distilled water
50 mL 0.2M phosphate buffer, pH=7.4

Lamprey ringer's solution, pH=7.2

Add the following constituents to 1L distilled water:

7.6 g NaCl
0.22377 g KCl
0.4066 g $MgCl_2$
2.383 g HEPES
0.294 g $CaCl_2$
0.901 g glucose

Osmium tetroxide(1% OsO_4)

- 2mL 4% OsO_4
- 2mL 0.225 sodium cacodylate buffer
- 4mL distilled H_20
- this yields 8mL of 1% OsO_4

Karnovsky's fixative

Immerse tissue in Karnovsky's:

16% Paraformaldehyde Solution:.....13mL
50% Glutaraldehyde EM Grade:.....5mL
0.2M Sodium Phosphate Buffer:.....50mL
Distilled Water:.....32mL

Final mixture is 2% Paraformaldehyde, 2.5% Glutaraldehyde and 0.1M Buffer.

Appendix 2

Table 11. Details of all larval sea lampreys used in the study of Chapter 2. "Animal code" refers to an internal coding system used for tracking the length and weight of each larval sea lamprey used in any study (including this one) within the Zielinski Laboratory.

Animal Code	Sex	Weight (g)	Length (cm)
L5003	n/a	2.30	11.8
L5103	n/a	1.74	10.4
L5203	n/a	1.76	11.3
L5303	male	1.92	11.5
L5403	n/a	1.22	10.1
L5503	female	2.64	12.5
L5603	female	2.26	10.7
L5703	male	2.0	10.0
L5803	female	2.08	11.6
L5903	Female	1.52	10.8
L6003	Female	2.42	12.1
L6103	male	1.63	10.7
L6203	male	1.78	10.4
L6303	Female	1.73	10.8
L6403	Male	1.38	10.1
L6503	Male	1.85	11.4
L6603	Female	1.85	10.5
L6703	Female	2.07	11.5
L6803	male	1.22	10.5
L6903	female	1.61	10.4
L7003	Female	1.54	11.0
L7103	Male	1.58	10.9
L7203	female	1.13	9.2
L7303	male	1.36	10.0
L7403	male	1.55	10.6
L7503	female	1.15	9.90
L7603	female	1.33	10.1
L7703	n/a	1.35	9.50
L7803	n/a	1.25	10.0
L3903	n/a	2.73	12.8
L4003	n/a	2.01	11.8
L4103	n/a	3.27	14.2
L4203	n/a	2.18	12.5
L4303	n/a	2.77	12.4
L4403	n/a	3.06	13.3
L4503	n/a	3.24	12.0

Animal Code	Sex	Weight (g)	Length (cm)
L4603	n/a	1.84	10.5
L4703	n/a	1.49	10.2
L4803	n/a	1.58	11.0
L4903	n/a	1.42	10.9

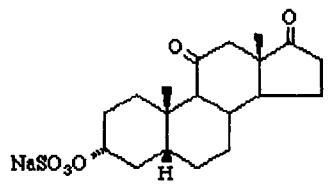
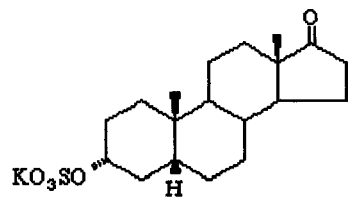
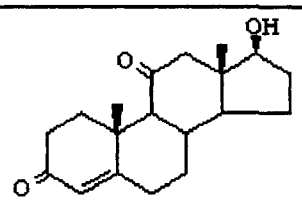
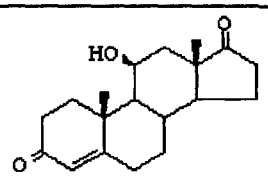
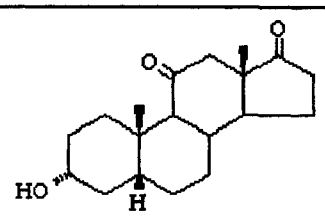
Appendix 3.

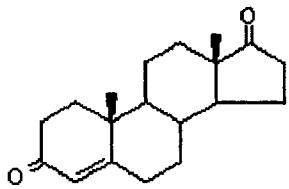
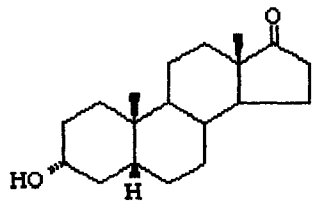
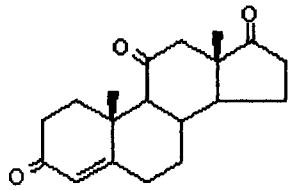
Table 12. Details of all male round gobies used in the study of Chapters 3 and 4.

Season Used and Study Used	Whole body mass (g)	Length (cm)	Total gonad mass (testis and seminal vesicle) (g)	Testis mass (g)	Seminal Vesicle mass (g)	Gonadal Somatic Index (gonad weight/ body weight*100)
July 2003						
Used for *in-vitro* testis and seminal vesicle incubation	44.2	14.5	0.77	0.565	0.205	1.74
Used for *in-vitro* testis incubation	51.5	16.5	0.832	0.35	0.482	1.61
Used for *in-vitro* testis incubation	47.7	15.5	0.88	0.80	0.08	1.84
Used for *in-vitro* seminal vesicle incubation	61.15	15.5	0.473	0.343	0.13	0.77
Histology: plastic sections (Chapter 3)	41.9	15.2	0.83	0.72	0.11	1.98
Histology: plastic sections (Chapter 3)	62.2	16.5	0.96	0.71	0.25	1.54
July 2002						
Used for *in-vitro* testis incubation	43.8	14.9	0.99	n/a	n/a	2.26
Used for *in-vitro* testis incubation	35.8	13.9	0.81	n/a	n/a	2.26
Used *for in-vitro* testis and seminal vesicle incubation	35.6	13.8	0.75	n/a	n/a	2.11
Used *for in-vitro* seminal vesicle incubation	40.1	13.8	1.05	n/a	n/a	2.62

Appendix 4

Table 13. Structures of steroids identified in Chapters 3 and 4. These structures were obtained from Steraloids Inc. (www.steraloids.com).

Steroid Name	Structure and molecular weight
11,17-dioxo-5β-androstan-3α-yl sulfate (11oxo-ETIO-s)	 MW = 406.47 g/mol
3α-hydroxy-17-oxo-5β-androstan-3α-yl sulfate (ETIO-s)	 MW = 408.59 g/mol
17β-hydroxyandrost-4-ene-3,11-dione (11-oxo-T)	 MW = 302.41 g/mol
11β-hydroxyandrost-4-ene-3,17-dione (11β-OH-Ad)	 MW = 302.41 g/mol
3α-hydroxy-5β-androstane-11,17-dione (11-oxo-ETIO)	 MW = 304.42 g/mol

Steroid Name	Structure and molecular weight
androst-4-ene-3,17-dione (Ad)	 MW = 286.41 g/mol
3α-hydroxy-5β-androstan-17-one (ETIO)	 MW = 290.44 g/mol
4-Androsten-3,11,17-trione (11-oxo-androstenedione)	 MW = 300.39 g/mol

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