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Studies of olfactory sensory neurons in two invasive fish species

Alyson Laframboise
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Studies of olfactory sensory neurons in two invasive fish species

By

Alyson J. Laframboise

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Studies of Olfactory Sensory Neurons in Tow Invasive Fish Species

by

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Declaration of Co-Authorship / Previous Publication

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows: this thesis also incorporates the outcome of a joint research undertaken in collaboration with Dr. Yogesh Katare under the supervision of Professor Barbara Zielinski. The collaboration is covered in Chapters 3, 4, 5, and 7 of the thesis. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-author was through the performance of the enzyme linked immunosorbant assay as it is described in these chapters.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from the co-author to include the above material in my thesis. I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

II. Declaration of Previous Publication

This thesis includes 3 original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*
Chapter 2	*Olfactory sensory neurons in the sea lamprey display polymorphism*	*Published 2007*
Chapter 3	*The effect of elevated steroids released by reproductive male round gobies (Neogobius melanostomus) on olfactory responses in females.*	*Accepted Jan 2011*
Chapter 4	*Female round gobies (Neogobius melanostomus) detect and discriminate between steroids released by male round gobies.*	*Submitted – under review*

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Abstract

Fishes widely employ the olfactory sense for communication in most aspects of their life. The olfactory system of fishes contains olfactory sensory neurons (OSNs) of three polymorphisms – ciliated, microvillous and crypt. This thesis describes various studies of the properties of OSNs, from the expression of polymorphisms to odour responses, receptor specificity and second messengers, in two species of invasive fish: the sea lamprey (*Petromyzon marinus*) and the round goby (*Neogobius melanostomus*). I describe, for the first time, the expression of ciliated OSNs displaying polymorphisms in the sea lamprey, an ancient jawless fish. In the round goby, I investigate the olfactory properties of several steroids that have been identified as released by the reproductive male (RM) round goby and are putative pheromones. Female gobies detect the novel steroids 11-oxo-etiocholanolone (11-O-ETIO) and 11-O-ETIO-3-sulfate, but not 11-O-ETIO-17-sulfate or 11-O-ETIO-3-glucuronide. In addition, these steroids act upon separate olfactory receptor mechanisms and are transduced via both cAMP and IP_3.

Female electro-olfactogram resposnes to methanol-extracted steroids from RM conditioned water increased following treatment of the RM with gonadotropin releasing hormone, but not saline. In addition, there was a correlation between female reproductive status (as measured by gonadosomatic index) and response to NRM (but not RM) urine.

We tested the possible modulation of olfactory responses by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). Perfusing $PGF_{2\alpha}$ directly over the olfactory epithelium had no effect on olfactory responses. Olfactory responses had a tendency to increase 40 minutes following injection with $PGF_{2\alpha}$, although this was not a significant change.

To investigate the possibility that RMs release pheromones with endocrinological effects, we measured female testosterone release following exposure to RM or NRM conditioned water and found that testosterone was unaffected by exposure to male odours.

This thesis describes olfactory properties for two fish species of ecological and taxonomic importance. We conclude that these studies of wild fish make important contributions to the field of fish olfactory biology, which is lacking in studies investigating core olfactory biological properties in fish of diverse taxonomic groups or in wild-caught populations.

Dedicated to my parents Alan and Brenda and to Martin for loving,

supporting and inspiring me in every aspect of my life.

Acknowledgements

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11-O-ETIO	11-oxo-etiocholanolone
11-O-ETIO-3-g	11-oxo-etiocholanolone-3-glucuronide
11-O-ETIO-17-g	11-oxo-etiocholanolone17-glucuronide
11-O-ETIO-3-s	11-oxo-etiocholanolone-3-sulfate
11-O-ETIO-17-s	11-oxo-etiocholanolone-17-sulfate
15-K-PGF2a	15-keto-prostaglandin $F_{2\alpha}$
17α,20β-P	17α,20β-dihydroxy-4-pregnen-3-one
17α,20β-P-s	17α,20β-dihydroxy-4-pregnen-3-one-sulfate
AC	Adenylyl cyclase
AD	Androstenedione
ELISA	Enzyme linked immunosorbant assay
EOG	Electro-olfactogram
ETIO	Etiocholanolone
cAMP	Cyclic adenosine monophosphate
CNG	Cyclic nucleotide gated
GPCR	G-protein coupled receptor
HPLC	High performance liquid chromatography
IP_3	Inositol-1,4,5-triphosphate
MS	Mass spectrometry
NRF	Non-reproductive female
NRM	Non-reproductive male
OE	Olfactory epithelium
OR	Olfactory receptor
OSN	Olfactory sensory neuron
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGE2	Prostaglandin E_2
PLC	Phospholipase C
RF	Reproductive female
RM	Reproductive male
sGnRHa	Salmon gonadotropin releasing hormone analogue
T	Testosterone
TAAR	Trace amine associated receptor
TCA	Taurocholic acid

Chapter 1:

Olfactory sensory neurons of fishes

1.1 General overview

Fishes rely strongly on their olfactory sense for virtually all aspects of their life, from finding food to avoiding predation, to finding and choosing a mate, and to identifying kin. Considering this, it is not surprising that fishes have a well-developed olfactory system that they use widely for communication. Fish communication is often accomplished via the use of pheromones, which are defined by Stacey and Sorensen (2006) as "*a substance, or mixture of substances, released by an individual, which evokes a specific and adaptive response in conspecifics, the expression of which does not require learning*". Specific types of pheromones are discussed further in section 1.7.

This thesis discusses studies of the olfactory sense and olfactory sensory neurons (OSNs) of two species of fishes – the sea lamprey (*Petromyzon marinus*) and the round goby (*Neogobius melanostomus*). I investigate properties of OSNs in these species including polymorphisms, receptor odour specificity and second messengers. These studies of OSNs in the round goby are unprecedented for a wild-caught fish species. Here, the overall interest was how the olfactory epithelium of female round gobies responds to compounds released by the reproductive males. We were working under the hypothesis that males release steroids which function as reproductive pheromones. I focus on responses to 5β- and 3α-reduced steroids released by reproductive males that are putative pheromones and on the urine and conditioned water that contains these compounds. In addition to testing for olfactory sensory responses, I investigate whether endocrine changes in the receiver affect these olfactory responses, and start to ask if these putative pheromones subsequently have biological activity that directly affects the endocrine system of the receiver.

The purpose of this introductory chapter is to review what is currently known about the olfactory epithelium (OE) in fishes in terms of structure and function of the OSNs.

1.2 Olfactory sensory neuron polymorphisms

The naris – the external opening to the environment – leads to the nasal cavity, which contains the OE. The OE is a pseudo-stratified ciliated epithelium consisting of bipolar OSNs, supporting cells and basal cells. Axons of OSNs exit the OE by passing through the basal lamina into the underlying lamina propria and join together to form the olfactory nerve, which transmits information to the olfactory bulb (located in the brain), where OSN axons form synapses with mitral cells, the second order neurons in the olfactory system. From the olfactory bulb, odour information is sent via the medial and lateral olfactory tracts to the telencephalon (reviewed by Laberge and Hara, 2001; Hansen and Reutter, 2004). It is within the OE that odours first bind to their receptors and olfaction – smelling – begins to take place. The olfactory signal transduction cascades are demonstrated in Fig 1.1 and Fig 1.2. Ultrastructural studies of the OE of fishes over the past 30 years or more have revealed that OSNs in fishes display polymorphisms that are tied to the chemical repertoire of these cells. Polymorphisms have been specifically described in many species including – but not restricted to – channel catfish, *Ictalurus punctatus* (Caprio and Raderman-Little, 1978; Muller and Marc, 1984); goldfish, *Carassius auratus* (Muller and Marc, 1984); other cyprinid fishes (*Aplochelius lineatus, Xiphophorus helleri*; Zeiske and Melinkat, 1976); and salmonid fishes (Thommesen, 1983). These polymorphisms differ in relation to their shape, position within the OE, and functionality (summarized in Table 1.1). The first type, the ciliated OSN, has a cell body located deep in the OE, near the basement membrane. Its dendrite is therefore long, and also quite thin, with a bulbous ending known as the olfactory knob, which bears cilia (reviewed by Hansen and Zielinski, 2005; Zielinski and Hara, 2007). In the OE of

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teleost fishes, ciliated OSNs are intermingled with the second morph, microvillous OSNs. The cell bodies of microvillous OSNs are located in the middle third of the OE, and they have mid-length, thick dendrites that end in microvilli. The two polymorphisms are seen even in rainbow trout embryos (*Salmo gairdneri*), with ciliated OSNs appearing 8 days earlier than the microvillous type (Zielinski and Hara, 1988). In addition to teleosts, the expression of both ciliated and microvillous sensory cells is seen in a primitive extant ray-finned fishes, the bichir (*Polypterus senegalus* and *P. ornatipinnis*; Zeiske et al., 2009) and sturgeons of the genus Acipencer (Zeiske et al., 2003).

The final OSN morph is the crypt cell (Hansen and Zeiske, 1998). It lacks a dendrite, and instead has a cell body located in the superficial OE and the longest axonal length passing through the OE of the OSN morphs. The crypt OSN gets its name from the crypt-like invagination at its apical surface which bares both microvilli and cilia (Hansen and Zeiske, 1998). The crypt cell appears to be widespread in teleost fishes (Hansen and Finger, 2000; Belanger et al., 2003; Castro et al., 2008; Bettini et al., 2009), sturgeons (Zeiske et al., 2003; Camacho et al., 2010), bichirs (Zeiske et al., 2009) and elasmobranchs (Ferrando et al., 2007; Ferrando et al., 2009).
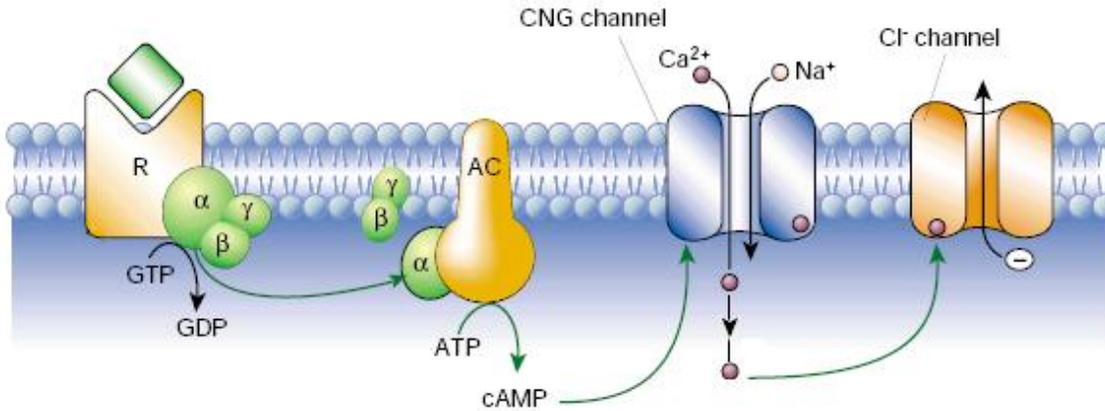
Fig 1.1: Summary of the olfactory signal transduction cascade involving cAMP as a second messenger. Upon binding of the odour molecule, the G-protein coupled to the receptor (R) is activated, releasing the α subunit which activates adenylyl cyclase (AC). AC, in turn, leads to an increase in cAMP which binds to the cyclic nucleotide gated (CNG) channel. Following opening of the CNG channel, positive ions flow into the cell, leading to action potential generation. Additionally, OSNs contain an unusually high intracellular Cl^- concentration, which flows out of a Ca^{2+} activated chloride channel to amplify the signal. The flow of ions creates the summed generator potential that we record as the elctro-olfactogram. Adapted by permission from Macmillan Publishers Ltd: [NATURE] (Firestein), copyright (2001).
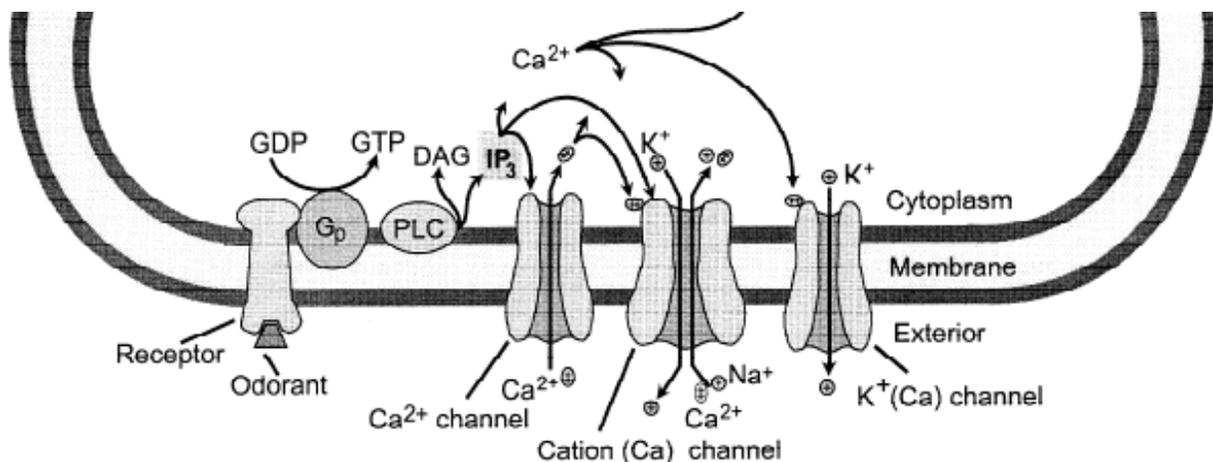
Fig 1.2: Summary of the olfactory signal transduction cascade involving IP_3 as a second messenger. Odour binding causes an increase in IP_3 via phospholipase C (PLC). The increase in intracellular IP_3 leads to the opening of Ca^{2+} channels and increased $[Ca^{2+}]$ activates non-specific cation channels and may activate a Ca^{2+}-activated K^+ channel. Modified with permission from Springer. Journal of Membrane Biology 181(2), copyright (2001).

The expression of OSN polymorphisms appears to follow an evolutionary pattern in those species that have been investigated. In an ancient jawless fish (superclass Agnatha), the sea lamprey (*Petromyzon marinus*), only ciliated OSNs are seen (Vandenbossche et al., 1995); in elasmobranchs, these sensory cells only bear microvilli (Takami et al., 1994; Ferrando et al., 2007; Schluessel et al., 2008; Ferrando et al., 2009; Ferrando et al., 2010); and the crypt cell appears in the OE (Ferrando et al., 2007; Ferrando et al., 2009). By the divergence of the ray-finned fishes, the OE was populated by all three OSNs (Zeiske et al., 2003; Zeiske et al., 2009; Camacho et al., 2010). Overall, in fishes displaying all three morphotypes, ciliated cells predominate, with microvillous OSNs being fewer in number (e.g. Thommesen, 1983). Generally, crypt cells tend to be rare (eg. Bettini, 2009), and in some species, do not appear in all specimens (Belanger et al., 2003). Considering their scarcity (e.g. Belanger et al., 2003), as well as the fact that their number may vary with season (Hamdani et al., 2008) and sex (Bettini et al., 2009), it is not all that surprising that the crypt cells escaped detection for so long.

Table 1.1: Summary of OSN morphotypes and their various properties. The dotted line in the first row designates the apical and basal surfaces of the olfactory epithelium (OE).

		Ciliated	Microvillous	Crypt Cell
OSN Morphotype				
Apical surface		Olfactory knob with cilia	Short olfactory knob with microvilli	Submerged crypt with microvilli and cilia
Dendrite		Thin	Thick	Absent
Cell body location in OE		Lower third	Mid region	Upper third
Taxonomy		Agnathans, teleosts	Elasmobranchs, teleosts	Teleosts
Odour Responses	Amino acids	Yes	Yes	Yes
	Bile acids	Yes	Unknown	Unknown
	Steroids	Unknown	Unknown	Unknown
Receptor Expression		OR-type	V2R-type	Unknown
G protein Expression		$G_{\alpha olf}$	$G_{\alpha q/11}$ / $G_{\alpha o}$ / $G_{\alpha i\text{-}3}$	$G_{\alpha q/11}$ / $G_{\alpha o}$
Second messengers		cAMP	IP_3	cAMP
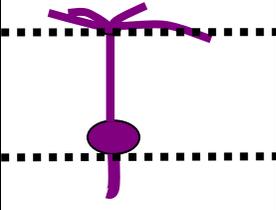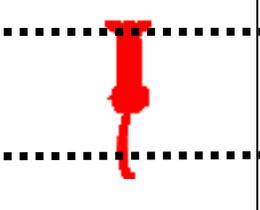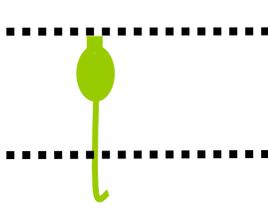
1.3 Olfactory sensory neurons – receptors

OSNs express olfactory receptors, the binding sites for odourous molecules (Buck and Axel, 1991). In fishes, as in mammals, the olfactory receptor is a seven transmembrane G-protein coupled receptor (GPCR), first described for fishes in channel catfish (Ngai et al., 1993b). Compared to mammals, which have about 1,000 genes encoding olfactory receptors (Buck and Axel, 1991; Parmentier et al., 1992), fishes have about 100 olfactory receptor genes (Ngai et al., 1993b). These receptors show no topography in the OE, rather they are randomly distributed throughout the OE (Ngai et al., 1993a).

In goldfish, two multigene olfactory receptor families have been cloned (Cao et al., 1998). These families, termed GFA and GFB, contain homologs of olfactory receptors (OR) found in mammals (Buck and Axel, 1991), and mammalian vomeronasal organ receptors (V2R;

Buck, 2000), respectively. These goldfish OR-type and V2R-type receptors are 7 transmembrane GPCRs as mentioned above. In fishes, ciliated OSNs express the OR-type receptors, so named because they resemble the ORs identified in mammals, while microvillous OSNs express the V2R-type receptors, which are similar to the receptors associated with vomeronasal organ of mammals (Cao et al., 1998; Buck, 2000; Hansen et al., 2004; Sato et al., 2005). In goldfish, OR-type and V2R-type receptors do not co-localize in the same cell morph (Hansen et al., 2004; Sato et al., 2005). To my knowledge, there is no published study about what type of receptor might be expressed in the crypt cell. In addition to the OR-type and V2R-type receptors, a third type of receptor has been identified in teleost fish – the V1R-type, which the authors named ora and identified in zebrafish (*Danio rerio*) OSNs (Saraiva and Korsching, 2007). In mammals the V1R and V2R receptors are quite different in terms of sequence similarity with each other and the ORs, and expression pattern in the vomeronasal organ (reviewed by Ma, 2007). Neurons expressing V1R or V2R receptors project to different areas of the mammalian accessory olfactory bulb, and respond to different classes of molecules (Leinders-Zufall et al., 2000; Ma, 2007). The final type of chemosensory receptor thus identified is the trace amine-associated receptor, which has been seen in many vertebrates, including zebrafish and *Fugu* (Gloriam et al., 2005), but has not yet been associated with any particular OSN morph.

Very little is known regarding olfactory receptors in non-teleost fishes. Few ancient fishes have the same genetic resources as the sea lamprey, which has a sequenced genome. Chemosensory receptor genes were detected in the sea lamprey, including 27 OR-type genes, 28 TAARs and 4 V1R-type genes; and all three gene families were expressed in the olfactory system of different sea lamprey life stages – parasitic and adult (Libants et al., 2009).

1.4 Olfactory sensory neurons – G-proteins

There have been two identified GPCR signal transduction cascades identified in the fish olfactory system: one stimulating cyclic adenosine monophosphate (cAMP) and one stimulating inositol-1,4,5-triphosphate (IP_3, Restrepo et al., 1990). The canonical signal transduction cascade in OSNs is summarized in Fig 1.1. As mentioned previously, the olfactory receptor is a GPCR, a G-protein coupled receptor. An olfactory-specific G-protein was identified in 1989 by Jones and Reed. This G-protein, named $G_{\alpha olf}$, has since been identified in the OE of several species of fishes. In fact, the expression of $G_{\alpha olf}$ is associated exclusively with the ciliated OSNs in teleosts (Belanger et al., 2003; Hansen et al., 2003; Hansen et al., 2004). In addition, $G_{\alpha olf}$ immunoreactivity has been seen in sea lamprey OSNs, which are all ciliated (Frontini et al., 2003). Microvillous OSN G-protein expression is species-specific, and these neurons never express $G_{\alpha olf}$. In goldfish, microvillous OSNs express $G_{\alpha o}$, $G_{\alpha q}$ or $G_{\alpha i-3}$ (Hansen et al., 2004), while in catfish, this morph expresses $G_{\alpha q/11}$ (Hansen et al., 2003), and in the round goby $G_{\alpha o}$ is associated with the microvillous cells (Belanger et al., 2003). In the shark (*Scyliorhinus canicula*), and rabbit fish (*Chimaera monstrosa*) which only have microvillous cells, OSNs seem to express $G_{\alpha o}$ only (Ferrando et al., 2009; Ferrando et al., 2010). Crypt cells express $G_{\alpha o}$ in round gobies (Belanger et al., 2003) and catfish (Hansen et al., 2003), and in the goldfish, crypt cells can express both $G_{\alpha o}$ and $G_{\alpha q}$ – the only published report of an OSN morphotype expressing more than one G-protein (Hansen et al., 2004).

1.5 Olfactory sensory neurons – second messengers and odour responses

As mentioned above, two second messenger pathways have been described in the olfactory system, one using cAMP and one using IP_3, which was first described by Restrepo and colleagues in channel catfish in 1993 (reviewed in Zielinski and Hara, 2007). Through

experiments using various physiological and histological techniques, these two second

messengers have been associated with the transduction of different odour classes. Table 1.2

summarizes research identifying the second messenger and OSN type associated with odour.

Table 1.2: Summary of second messengers and OSN morphotypes associated with different odour classes based on electro-physiological and immunohistochemical studies in fish.

Odour Class	Second Messenger		OSN morph
	cAMP	IP$_3$	
Amino acids	Catfish (Hansen et al., 2003) Goldfish (Rolen et al., 2003) Mackerel (Vielma et al., 2008)	Catfish (Hansen et al., 2003) Goldfish (Rolen et al., 2003) Zebrafish (Michel et al., 2003; Michel, 1999) Atlantic salmon (Lo et al., 2003)	**Microvillous** Catfish (Hansen et al., 2003) Rainbow trout (Sato and Suzuki, 2001) **Crypt** Mackerel (Vielma et al., 2008)
Bile salts	Catfish (Hansen et al., 2003) Goldfish (Rolen et al., 2003) Zebrafish (Ma and Michel, 1998; Michel 1999)	Atlantic salmon (Lo et al., 1994)	**Ciliated** Catfish (Nikonov and Caprio, 2001; Hansen et al., 2003) Goldfish (Rolen et al., 2003) Rainbow trout (Sato and Suzuki, 2001)
Pheromones or steroids	Goldfish (Sorensen and Sato, 2005)	Not observed	Unknown

Generally speaking, amino acid odours – which represent food odours – are transduced via

cAMP and/or IP$_3$. These responses appear to take place in microvillous OSNs and therefore

involve G-proteins other than G$_{\alpha olf}$ (e.g. G$_{\alpha o}$ or G$_{\alpha q}$). The single study of crypt cells found that

these responded only to amino acids and that these responses were mediated via cAMP (Vielma

et al., 2008). With the exception of the Atlantic salmon, bile salt odours (proposed to function in

social interactions) are processed by ciliated OSNs using cAMP (via G$_{\alpha olf}$).   Previous

investigation of second messengers responsible for transducing pheromones (in this case a blend

of steroids), found that cAMP transduced olfactory response to a mixture containing sex steroids

(Sorensen and Sato, 2005).

The second messenger cAMP binds to and activates a cyclic nucleotide gated channel, the opening of which allows an influx of positive ions. This flow of ions creates the generator potential that we record as the electro-olfactogram, which is described further in section 1.8.

1.6 Olfactory sensory neurons – summary

In the OE of fishes, there exists a relationship between form and function of the OSNs. The three OSN morphotypes (ciliated, microvillous and crypt) vary in their shape and position within the epithelium. Along with this, they express different receptors (OR-type and V2R-type, as well as V1R-type and TAARs, though these last two have not been specifically associated with particular morphs) which are coupled to different G-proteins. These OSN types also transduce different odour classes and utilize different second messengers to do so. Studies of OSNs have revealed these characteristics in several species of fishes, but additional information – particularly for more diverse taxonomic groups of fishes – is still necessary to define the complete form and function of OSNs.

1.7 Primer and releaser pheromones

Pheromones, defined in section 1.1., can be further divided into two types, based on the receiver's response. Pheromones causing no behavioural effect, but instead having a physiological or endocrinological effect are termed primer pheromones, while pheromones which do elicit overt behavioural effects in the receiver are termed releaser pheromones (Wilson and Bossert, 1963). In fishes, as in other animals, olfactory responses to releaser pheromones can recorded from the OSNs in the OE using electro-olfactogram (EOG, explained in section 1.8). Primer pheromones, however, do not necessarily have to evoke an EOG response – the EOG being indicative that the fish can smell the odour. The best known example of this is the synchronization of the menstrual cycles of female humans as observed by McClintock (1971).

The females in this study could not smell the primer pheromone that was being released, but it had a physiological effect. This classic example indicates that pheromones can function without conscious detection, but similar examples do not exist in fishes because researchers tend to measure the potential of a putative pheromone by its evoked EOG response. However, Chung-Davidson and colleagues (2008) found that in the goldfish, releaser pheromones also had neurogenic effects, as demonstrated by an increase in the number of cells dividing in the diencephalon of the brain.

1.8 The Electro-olfactogram (EOG)

The EOG is a well-known tool for the study of olfaction in fishes particularly (Silver et al., 1976). This electrophysiological technique records responses to odours as changes in the field potentials recorded from neuron ensembles. This response is a slow, DC recording of summed generator potentials, which are graded responses to stimuli. EOG responses to odours occur in a dose-dependent manner and are typically used to determine detection thresholds for odours. Potency of an odour can be inferred from EOG recordings using the response magnitude in millivolts (i.e. the odour-evoked change in the field potential measured in millivolts) or the normalized response (i.e. the odour response in millivolts expressed relative to the response to a pre-determined standard odour). In addition, the ability to block or reduce EOG responses using odours or pharmacological agents allows for the discernment of certain olfactory properties such as the receptor specificity or the second messenger involved. Thus, the EOG is a very useful tool in the study of olfaction.

1.9 Olfactory studies using wild fish populations

The work described in this thesis all involves fish caught from wild populations that have not been bred in the lab, which is rare in the olfactory field, particularly in respect to electro-

olfactogram (EOG) studies. Working with a wild population of round gobies presents a number

of difficulties. For example, EOG recordings cannot be taken after about early to mid-October

and behavioural responses to odours also decrease after the summer for reasons yet unknown.

EOG recordings have only been reported for a small number of wild-caught fishes. The

EOG recordings from the sea lamprey are well-known and have been published for years (Li et

al., 1995; Siefkes et al., 2003). Recently, a study employing EOG recordings to investigate

putative pheromones in wild-caught field specimens of peacock blenny (*Salario pavo*) has been

published (Serrano et al., 2008). In addition to these species, EOG studies have been published

using wild black bullhead catfish (*Ameiurus melas,* Dolensek and Valentincic, 2010) and wild-

caught hammerhead shark (*Sphyrna lewini,* Tricas et al., 2009).   Considering the vast number of

EOG studies that have been published using fishes, this is a very small proportion – about 14%

of results in one Pubmed search (though it was not exhaustive), and about 50% of these were sea

lamprey. This indicates that that not only is the overall number of EOG studies of wild species

low, but the number of species represented in published studies is even lower because a large

proportion of them use the sea lamprey.

1.10 Non-reproductive females in the study of pheromones

All of the studies in this thesis used non-reproductive females, and this fact must be

addressed. Although reproductive females are ideal for EOG testing of putative pheromones, the

difficulty in obtaining these females and our inability to maintain their reproductive status once

brought in to our facility, make them an unreliable study subject. Female round gobies are

classified as "reproductive" if they have a gonadosomatic index (GSI) of 8% or greater (Belanger

et al., 2004) or "non-reproductive if the GSI value is less than 8%. The GSI is a measure of

gonad weight expressed as a percentage of total body weight, and in most fishes, is the most

common means of assessing reproductive status of both males and females. In females, a high GSI typically indicates that the female's ovaries are full of eggs. Although reproductive female round gobies were seen to display greater EOG responses to male odours than non-reproductive females (Belanger et al., 2004), non reproductive females detect the same odours, though with a lower response magnitude. Also, male and female round gobies did not show a difference in EOG responses to steroid odours (Murphy et al., 2001), though this study did not use reproductive individuals. Overall, there is no evidence that an olfactory receptor for a pheromone might only be expressed during the fish's reproductive stage. The number of olfactory receptors may increase with sexual maturity (Lai and Hong, 2010), which may lead to an increased olfactory response, but there is no reason to believe that the response would be absent in non-reproductive fish. Finally, as our work is part of an overall project to develop a pheromone trapping method for the round goby, the ability to attract and trap all life phases of this invasive fish is helpful.

1.11 Overview of thesis contents

The chapters contained in this thesis seek to add to this body of research by investigating OSN properties in two vastly different, but ecologically and phylogenetically important species of fish – the sea lamprey and the round goby. Both of these species are invasive to the Great Lakes regions. In addition, the sea lamprey is an ancient jawless Agnathan fish, while the round goby is a fish of the order Perciformes, a highly derived and extremely diverse order of fishes.

In Chapter Two we investigated OSN polymorphisms in the sea lamprey. We hypothesized that OSNs are a conserved evolutionary trait in fishes, and that polymorphisms would be present in some form in Agnatha. Although the sea lamprey has only ciliated OSNs,

these neurons could display morphologies similar to those seen in other fishes, in terms of somata location and dendrite length and width.

Figure 1.3 outlines visually the round goby studies contained in this thesis, beginning with testing whole extracts of male conditioned water, to testing the identified compounds in the extracts and on from there. Table 5.1 provides a summary of the steroids tested in this thesis, including their site of release and whether they are detected by EOG.
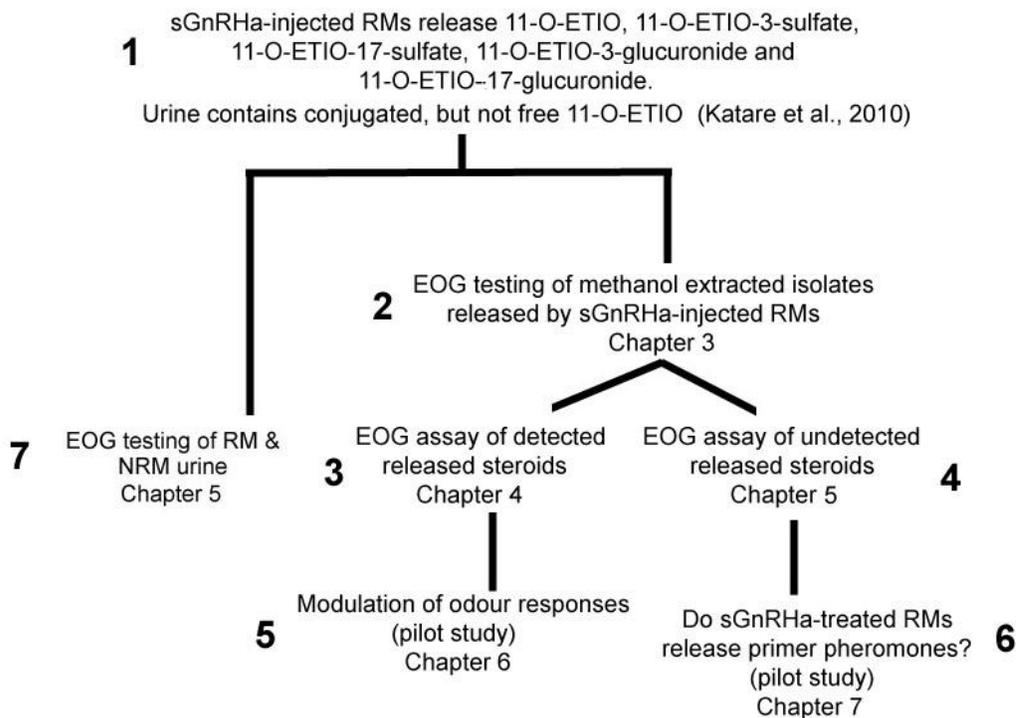
Fig 1.3: Flow chart outlining the flow of round goby studies in this thesis. Part 1 is the paper published by Katare et al. (2010) identifying released steroids that are putative pheromones. Part 2 concerns the EOG testing of the methanol extracted isolates that were prepared as part of the Katare paper. From there, parts 3 and 4 concern EOG testing of detected and undetected steroids identified by Katare et al. Part 5 relates to the possible modulation of odour responses while part 6 seeks to investigate whether undetected steroids might have primer pheromone function. Finally, part 7 is the EOG testing of urine collected from RM and NRM round gobies. RM = reproductive male; NRM = non-reproductive male; sGnRHa = salmon gonadotropin releasing hormone analogue.

We had previously seen that by treating male round gobies with gonadotropin releasing hormone, we could increase the amount of putative pheromones released into the water (Katare

et al., 2011). We hypothesize that the olfactory system of round gobies has evolved specific strategies for reproduction involving the use of pheromones. We predicted that the female smells the novel steroids being released by RMs (Fig 1.2), and that the signal transduction cascade functions in a specific manner for handling these compounds.

In chapter three, we specifically hypothesized that we would see an increased olfactory response from females in response to the methanol extracts of conditioned water containing more of the identified putative pheromones.

Chapter four describes the olfactory properties of the detected (i.e. "smelled") putative steroid pheromones identified in the methanol-extracted conditioned water and urine of the male round goby. In this chapter we described olfactory thresholds, receptor specificity and second messengers for these putative pheromones.

Chapter five is an investigation of other identified putative steroidal pheromones which we classify as not detected by the OE. In addition, we tested female olfactory responses to male urine, a source of substituted steroids which may be pheromones. We hypothesized that females would exhibit a greater response to urine collected from reproductive males compared to non-reproductive males.

Chapter six describes a pilot study investigating the possible modulatory effects of prostaglandin $F_{2\alpha}$ on OSNs. We were interested in whether olfactory responses might be modulated by prostaglandin $F_{2\alpha}$ which is associated with ovulation in female fishes. It is possible that undetected steroids might be detected during ovulation if the OSNs become more sensitive during reproduction. Based on previous literature, we expected to see an enhancement in olfactory responses recorded from female round gobies treated with prostaglandin $F_{2\alpha}$. This preliminary study was not completed in full due to time constraints.

Finally, in chapter seven we sought to investigate the possible endocrine effects of male round gobies on females. Steroids released by males that are not detected by the female OE may still function as primer pheromones, having an effect on the physiological state of the female. For this pilot experiment, females were exposed to male goby conditioned water with the expectation that we would see an increase in the female's release of steroid hormones. We only performed a pilot study because we were initially disappointed not to see a change, and because we did not measure the appropriate steroids.

Overall, I describe the OSNs of these species in terms of morphology (sea lamprey), specificity of receptors for putative pheromones (round goby) and second messengers (round goby) as well as other properties of the round goby olfactory repertoire and biology, such as other putative effects of released steroids on the receiver.

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Chapter 2:

Olfactory sensory neurons in the sea lamprey display polymorphisms

2.1 Introduction

In terrestrial vertebrates such as mammals, the olfactory system is composed of two functionally and physically distinct subsystems: the main olfactory system and the vomeronasal system. Each contains sensory neurons of different morphologies: ciliated sensory neurons which are present in the main olfactory system and microvillous sensory neurons which are present in the vomeronasal system [1,2]. It is established in mammals that these ciliated and microvillous cells differ functionally, with the different cells responding to different odour classes [2,13]. However, the evolutionary origin of these subsystems is unknown. A single morphotype, the ciliated olfactory sensory neuron (OSN) has been reported in lampreys [18,19] - ancient jawless fish phyletically removed from modern (teleost) fishes. The lampreys, including the sea lamprey, (*Petromyzon marinus*) occupy an important location at the base of the vertebrate evolution.

All fishes lack a distinct vomeronasal system for detecting social cues such as pheromones; however there are parallels between the mammalian vomeronasal and main olfactory system and the teleost fish olfactory system. Teleosts have three distinct morphotypes of OSNs intermingled in one olfactory epithelium: ciliated OSNs, microvillous OSNs and crypt receptor cells [10,8,14]. In teleosts, OSN morphotypes are distinguished by the location of their somata within the depth of the olfactory epithelium and the resulting length of their dendrites; both features lead to characteristic shapes. The ciliated OSNs have basally situated somata and long, thin dendrites while microvillous OSNs have an overall stubby, fusiform shape, somata located midway in the epithelium, and intermediate-length dendrites with microvilli [14]. The entire crypt cell is located apically, is egg-shaped and contains an invagination which is filled with cilia that do not breach the surface of the epithelium [10,11]. The crypt cell is exclusive to

fishes, but appears to be a common feature amongst both bony fishes and elasmobranchs [3]. In fishes, there is also great deal of evidence suggesting that these neurons differ functionally, including their differential olfactory bulb projections [14,15,6], molecular properties [16,7] and physiological properties [17,9,5]. This indicates that though these cells may not be spatially distinct in the periphery as in the mammalian system, there may be a functional distinction related to the morphological and biochemical properties of the cells. If this is the case, then evidence points to partitioning of the olfactory system in the processing of odour classes being a highly conserved trait.

Using retrograde tract tracing, we sought to identify OSN polymorphisms in the olfactory epithelium of the sea lamprey. The peripheral olfactory organ of lampreys comprises of the olfactory epithelium [18] and the accessory olfactory organ, which may also contain OSNs [4,20]. Additionally, as the sea lamprey exhibits a complex life cycle characterised by drastic physiological, anatomical and behavioural changes, we sought to elucidate differences in OSN distribution and morphology in two different life stages of the sea lamprey: metamorphic stage VII (post-metamorphosis but prior to parasitic feeding) [21], and the reproductively mature adult lamprey. These stages were chosen because they represent periods in the life cycle where the animal is driven by two very different biological needs: feeding and mating. The reproductively mature adult is the terminal life stage of the sea lamprey, and during this stage there is no sign of cell proliferation in the peripheral olfactory organ [12]. We employed two methods of retrograde labelling in the metamorphic stage VII lamprey specifically: biocytin tracing uses an *ex vivo* tissue preparation to investigate labelling in live tissue and DiI carbocyanine tracing uses fixed tissue.

2.2 Materials and Methods

Reproductive adult (N=16, June-October, 2006) and metamorphic stage VII (N=10, November 2005-April 2006) sea lampreys used in this study were obtained from the Hammond Bay Biological Research Station. All sea lampreys were collected from wild populations in the Great Lakes region. Initial experiments were conducted on metamorphic lampreys in the autumn and winter, when the reproductive phase was not available for study. In accordance with the Canadian Council on Animal Care, fish were anaesthetised in 0.05 g/l MS222 (tricaine methanesulfonate; Argent Laboratories, WA, USA), decapitated and the olfactory epithelium and brains exposed. Initially, two retrograde labelling techniques were used on the metamorphic phase lampreys: 1) the *post mortem* lipophilic tracer 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanineperchlorate (DiI), and 2) biocytin dye loading of live tissue. Both DiI (N=5) and biocytin (N=5) neuronal labelling strategies from these initial experiments in the metamorphic phase animals yielded identical polymorphic characteristics. We chose to stay with the biocytin labelling for the remaining metamorphic and adult samples because tissue was available for analysis for an extended period with this technique.

For carbocyanine labelling of *post-mortem* tissue, metamorphic stage VII stage sea lampreys (N=5) were anaesthetised and decapitated, and their brains were rapidly exposed and fixed in 4% paraformaldehyde. Following fixation overnight, the rostral portion of the olfactory bulb was lesioned and small crystals of the lipophilic tracer 1,1-dioctadecyl-3,3,3,3-tetramethylindocarbocyanine perchlorate (DiI) (Invitrogen, Canada) were inserted into the lesions. The tissue was then incubated in 4% paraformaldehyde at 37°C for 9 days allowing for the diffusion of the dye. Tissue was then sectioned at $40 - 50$ μm on a vibratome and then

imaged using a Bio Rad 1024 confocal microscope system. Stacked images were acquired and displayed as Z-series projections.

For biocytin labelling, the exposed brain was bathed in cold lamprey Ringer's solution (130 mM NaCl, 2.1 mM KCl, 2.6 mM CaCl2, 1.8 mM MgCl2, 4 mM HEPES, 4 mM dextrose, 1 mM NaHCO3, pH 7.4) and the medial rostral portion of the olfactory bulb was lesioned using an insect pin (Fig. 2.1) and 4% biocytin in 0.05 M tris buffer was injected into the lesion using a glass micropipette. The general areas where lesions were made are main entry points of axons into the olfactory bulb, and were chosen in order to maximise the number of axons able to take up the dye. The tissue was incubated for 4 hours at 7°C in cold oxygenated Ringer's solution replenished via gravity feed and aspiration. Following incubation, the tissue was fixed in 4% paraformaldehyde (in 0.1 M phosphate buffer) overnight, cryoprotected and cryostat (Microm) sections were taken at 14 − 16 μm. For secondary detection, slides were incubated in Alexa-substituted streptavidin (1:100; Invitrogen, Canada) for 2 hours. The sections were imaged on a Bio Rad 1024 confocal microscope system. Stacked images were acquired and displayed as Z-series projections.

OSN morphology was classified based on the depth of the soma within the olfactory epithelium as previously described [5]. To determine depth, the olfactory epithelium was divided into 5 arbitrary horizontal layers of approximately equal size, the most apical layer being layer 1 and the most basal being layer 5 (Fig. 2.2A, Fig. 2.3A). With this criterion, OSNs were grouped into one of three overall types [14]: tall OSNs were those cells whose somata were located in the deeper layers (3 or 4) of the epithelium, intermediate OSNs were those with somata in layer 2, and those cells with somata in layer 1 (the most superficial layer) were classified as short OSNs.

2.3 Results

Though all OSNs in the lamprey are ciliated [18,19], these cells exhibited morphotypes similar to all three OSN types seen in teleost fishes. Axonal processes extended to the base of the olfactory epithelium in all morphotypes. Tall OSNs were recognisable by their basally-located somata and their long dendrites, and were similar to the ciliated OSNs of teleosts. Intermediate OSNs had somata located in the middle layers of the epithelium and a fusiform shape similar to that of teleostean microvillous OSNs. Short OSNs were situated very apically and had an obvious egg shape and were similar to crypt receptor cells. The tall OSN was the dominant morph, and was ubiquitous throughout the epithelium. All three morphotypes were seen in both life stages examined and were the same regardless of the dye application shown in Fig. 2.1.
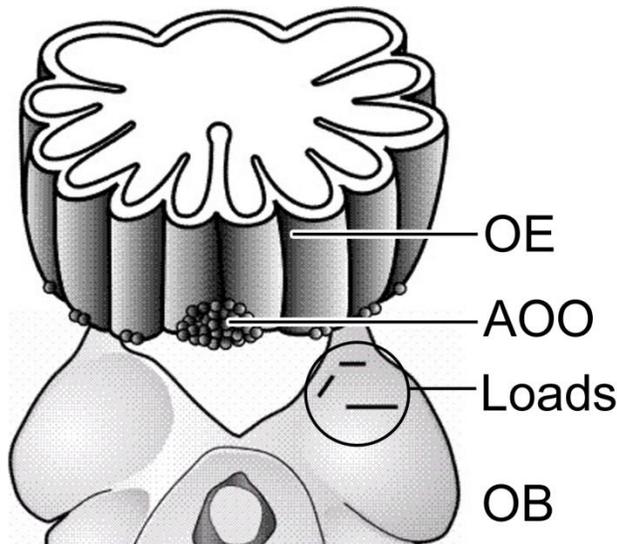
Fig. 2.1. A schematic diagram showing the location of the dye tracer loads in the primary olfactory pathway of the sea lamprey. Olfactory epithelium (OE) lines lamellae, and follicles of the accessory olfactory organ (AOO) are located ventrocaudal to the olfactory epithelium. The dye loads filled lesion sites made at the rostral edge of the olfactory bulb.

Loads from all three sites at the rostral edge of the olfactory bulb filled OSNs dispersed throughout the olfactory lamellae, with no apparent pattern attributable to the large lesions in

either life stage. Additionally, no differential distribution of OSN morphotype was observed in either life stage or from any loading location, with the more rare intermediate and short OSNs occurring in all parts of the olfactory epithelium.

The cell bodies of tall OSNs were situated within layers 3 or 4 of the epithelium and the long dendrites reached the epithelial surface, where the high, prominent bulbous extension of the dendrite known as the olfactory knob projected cilia into the olfactory mucosa (Fig. 2.2A). Within the tall OSN population in the adult stage lamprey, there were two types - the tall thin OSN and the tall thick OSN - that were seen with enough regularity to question whether they may also represent different morphologies. The tall thin OSN had a dendrite approximately half the relative width of the cell body (Fig. 2.2A, thin arrows). The tall thick OSN had a dendrite the same relative width as the cell body (Fig. 2.2A, thick arrows). The tall OSNs in the metamorphic stage VII lamprey were generally the thin type, displaying oval cell bodies and slender dendrites ending with the olfactory knob and often cilia were present (Fig. 2.2B). Cell counts were not performed, but generally the tall OSN was the most frequently observed morphotype in both life stages.

The intermediate OSNs displayed obvious axons, olfactory knobs and cilia (Fig. 2.3A). As is seen in the microvillous OSNs of teleosts, the intermediate OSNs had a stubby, slightly-fusiform shape, with the cell body located in layer 2 of the epithelium. Compared to the tall OSNs, the intermediate OSNs had lower, less obvious olfactory knobs (Fig. 2.3A, 2.3B, arrowheads), and a more tapered shape. The shape and location of the intermediate OSNs were the same in the metamorphic stage VII and adult stages of the sea lamprey (Fig. 2.3).

The short OSNs displayed the egg-shape characteristic of the crypt cells of teleosts [8] and the elasmobranch *Scilyorhinus canicula* [3] and also lacked dendrites due to their position in

the most superficial layer of the epithelium (Fig. 2.4A), as is the case in teleosts. The tops of

these short, rounded OSNs did not breach the surface of the epithelium, and often these cells had

a single, slender projection which remained embedded in the olfactory epithelium (Fig. 2.4A,

2.4B, asterisks). The short OSNs in the metamorphic stage VII lamprey were the same as those

of the adult stage lamprey (Fig. 2.4B), being situated in the most apical layer and showing the

single, submerged projection. We did notice some labelled cells located in layer 1 of the

epithelium which did not have the rounded, egg-shape characteristic of the crypt receptor cell of

teleosts. Rather, these short cells had tapered bottoms and their apical end sometimes extended

into the mucociliary matrix (data not shown).
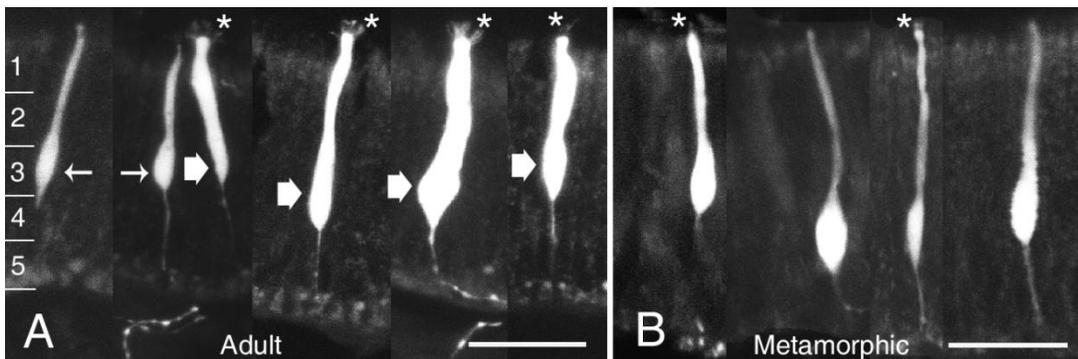
Fig. 2.2. Tall OSNs labelled with retrograde tracers in the adult and metamorphic stage VII sea
lamprey.
A. The division of the epithelium used for analysis is shown, demonstrating the 5 epithelial
layers. Tall OSNs in the adult stage had somata located in layer 3 or 4 of the olfactory
epithelium and high, prominent olfactory knobs. There were two subtypes of the tall OSN
morphotype: the tall thin (thin arrows) and tall thick OSNs (thick arrows). Cilia of many of these
cells are evident (asterisks).
B. Tall OSNs in the metamorphic stage VII sea lamprey with somata in layer 3 or 4 of the
epithelium. Scale bars = 50 μm.
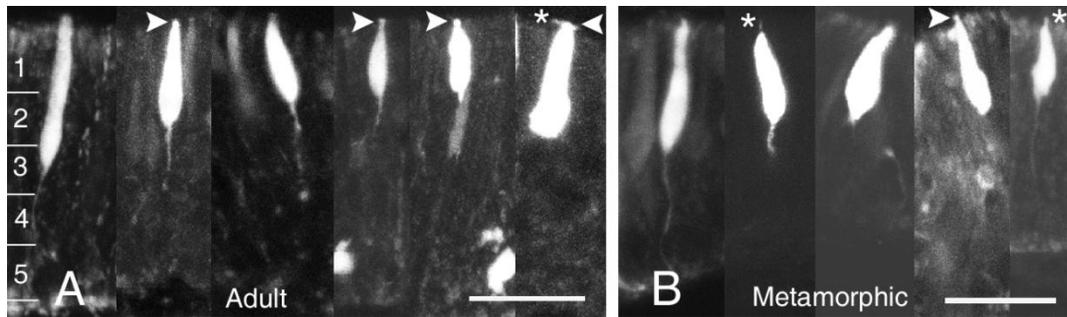
Fig. 2.3. Intermediate OSNs labelled with retrograde tracers in the adult and metamorphic stage VII sea lamprey.
A. Intermediate OSNs in the adult stage. The small and relatively flat olfactory knobs, were clearly evident (arrowheads) and displayed cilia (asterisk).
B. Intermediate OSNs in the metamorphic stage VII sea lamprey. These OSNs are the same as the intermediate OSNs of the adult stage sea lamprey. Olfactory knobs (arrowhead) and cilia (arrows) are apparent in some. Scale bars = 50 μm.
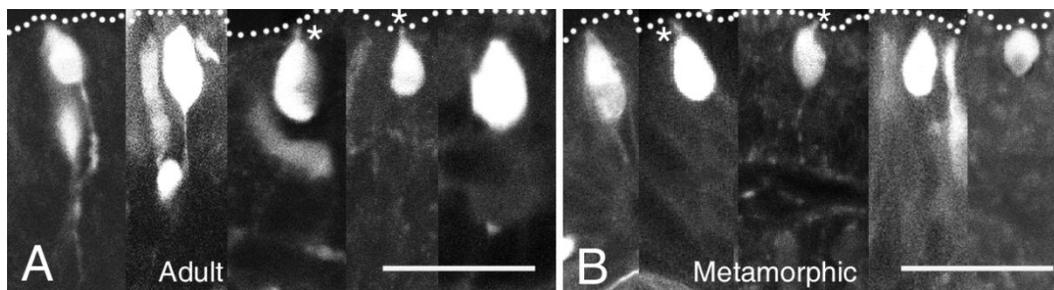
Fig. 2.4. Short OSNs labelled with retrograde tracers in the adult and metamorphic stage VII sea lamprey. The surface of the olfactory epithelium is indicated by a dotted line.
A. Short OSNs in the adult stage were egg-shaped and showed single apical projections (asterisks) which were submerged within the epithelium.
B. Short OSNs in the metamorphic stage VII sea lamprey, which also had the submerged projection which does not extend into the olfactory mucosa (asterisks). Scale bars = 50 μm.

2.4 Discussion

This study demonstrates that OSNs in the sea lamprey exhibit polymorphisms similar in many ways to OSNs seen both in teleost fishes. In addition to having the typical OSN morphotype − the tall OSN, the sea lamprey has OSN morphotypes similar to both the microvillous OSN and the crypt receptor cell seen in teleosts. The sea lamprey is an early vertebrate phyletically removed from modern fishes, the olfactory system of which nonetheless

shows many similarities with that of teleosts. This provides strong evidence for the evolutionary conservation of OSN polymorphisms and the conservation of the crypt cell. Considering the many similarities in the olfactory system of fishes, it is highly likely that these are indeed crypt receptor cells, given their characteristic shape and position in the epithelial layer, as well as their scarcity in all species that contain this cell type. We cannot completely rule out the possibility that these different morphs may be different developmental stages of the same type of cell; however, all three morphotypes were seen in both lamprey life stages examined and if they were related to age, we would expect to see differences in the adult sea lamprey which does not contain proliferating cells in the olfactory epithelium [12]. Both the lack of cellular proliferation in the adult stage and the close similarities seen in different piscine olfactory systems support our hypothesis that these are in fact different cell types. Table 1.1 demonstrates the functional differences between different OSN morphotypes in terms of G-protein expression and second messengers used in signal transduction cascade. These are also related to which odours may be processed by different OSN morphotypes. It may be that fishes evolved a way to process different odours based on the OSN type, and that this ability emerged as early as the sea lamprey. In adults, the driving force behind behaviour is the need for the animal to reproduce. In juvenile stages, it is the need to feed. These two basic functions are related to specific odours and the relatively small behavioural repertoire of the sea lamprey may have facilitated the evolution of OSN morphological subtypes that respond to particular odour classes considering the lamprey is strongly driven by only a relatively few odours. The sea lamprey demonstrates polymorphous OSNs similar to those seen in modern fishes, indicating that OSN polymorphism is an evolutionary conserved trait.

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Chapter 3:

The Effect of Elevated Steroids Released by Reproductive Male Round Gobies

(*Neogobius melanostomus*) on Olfactory Responses in Females.

3.1 Introduction

Many fishes use hormonal products released into the water as reproductive pheromones (Stacey et al., 2003). Gonadotropin releasing hormone (GnRH) is an important step in the production and release of steroidal hormones, as it stimulates the release of luteinizing hormone, which is responsible for stimulating gonadal steroid release (reviewed by Zohar et al., 2010). Many studies have investigated the effect that GnRH has on circulating plasma steroid levels, but it has rarely been demonstrated that increased GnRH can lead to an altered chemical signal that affects the response of opposite-sex conspecifics through increased release of steroidal compounds functioning as pheromones. Serrano et al., (2008) showed that treating male peacock blennies (*Salaria pavo*) with 11-ketotestosterone increased female olfactory responses to extracts of the gonads and anal glands. The olfactory system of the round goby (*Neogobius melanostomus*) responds to steroidal compounds (Stacey et al., 2001), and reproductive females showed an increased olfactory response to water from reproductive males versus non-reproductive males (Belanger et al., 2004). Katare et al. (2011) observed that following injection with salmon GnRH analogue (sGnRHa), male round gobies increased the release of several steroids into the water, including 11-oxo-etiocholanolone (11-O-ETIO) and 11-O-ETIO-3-sulfate (11-O-ETIO-3-s), which are novel fish steroids and putative pheromones in this species. Using electro-olfactogram recordings of female round gobies, I was interested in seeing how a surge of sGnRHa would affect the olfactory signal being released by the males.

3.2 Methods and Materials

3.2.1 Experimental Animals

Round gobies were angled from the Detroit River and Lake Erie in Windsor and Leamington Ontario, respectively. Fish were separated by sex and held in the animal facilities at

the University of Windsor in flow-through aquaria with dechlorinated municipal tap water at ambient temperatures on a 16:8 photoperiod. Fish were fed commercial food once daily (Aquatic Eco-systems, Apopka, FL, USA). Generally, fish were held for a maximum of one month. All experimental procedures conformed to CCAC guidelines.

3.2.2 Generation of Methanol Extracted Male Water

The procedure for generating methanol extracts of water that held reproductive males (RM) has been described elsewhere (Katare et al., 2011). Each RM goby (chosen on the basis of secondary sexual characters; Belanger et al., 2004) was isolated in 1 L of dechlorinated water which was collected after 4 hours ("pre-injection"). The RMs were injected with sGnRHa (Ova-RH, Syndel Laboratories, Qualicum Beach, BC, Canada) or 0.9% saline, immersed into fresh dechlorinated water (1 L) for 16 hr, and this water was collected ("post-injection"). Steroids were extracted from the water (1L) by passing through C18 cartridges (Sep-Pak, Waters, Milford, MA, USA) and eluting with 5 ml of methanol. Stock solutions of methanol extracted steroids were produced by diluting 100 µL of extract into 10 mL of decholorinated water to approximate the original concentration released by RMs. Individual stocks were diluted 100x further in dechlorinated water for EOG testing. A custom immunoassay was used to measure the amount of immunoreactive free and substituted 11-O-ETIO in the methanol extracts (Katare et al., 2011). Stock solutions (containing 100 µL extract) prepared from pre-injection extracts contained 11-O-ETIO immunoreactivity that averaged 0.085 ± 0.026 ng. In stock solutions prepared from post-injection extracts from saline and sGnRHa treated males the values for 11-O-ETIO immunoreactivity were 0.15 ± 0.092 ng and 1.29 ± 0.34 ng, respectively. All ELISA analyses were performed by Dr. Yogesh Katare.

3.2.3 Electro-olfactogram (EOG) Recordings

I recorded summed generator potentials from the surface of the olfactory epithelium using a recording procedure adapted from Murphy et al. (2001) and Bélanger et al. (2004). Non-reproductive female gobies were anesthetized, immobilized and placed in a recording trough with water containing anesthesia perfusing the gills. Glass capillary electrodes were placed in the anterior naris (recording electrode) and on the surface of the skin near the naris (reference electrode). Dechlorinated municipal tap water (used as background water) was constantly dripped over the olfactory epithelium via a tube positioned over the posterior naris. Odours were introduced into this water flow to avoid mechanical stimulation, as the flow of background water did not induce mechanical stimulation of the OSNs. The standard odour, 10^{-5} M L-alanine (Sigma-Aldrich, Oakville, ON, Canada) was delivered periodically to monitor the stability of the recording. We recorded responses to pre- and post-injection extracts. EOG responses were recorded in millivolts (mV) and normalized by dividing them by the average response to L-alanine. I used a data acquisition system (PowerLab, ADInstruments, Colorado Springs, CO, USA) and computer to record and analyze the data. Student's t-tests were used to analyze for statistical differences between pre-and post-injection extracts for the two treatments. Data were analyzed with SigmaStat 3.5 (Systat, San Jose, CA, USA).

3.3 Results

Methanol extracts of RM round goby conditioned water elicited robust olfactory responses from female round gobies (Fig. 3.1a). Females had a greater EOG response to the methanol extracts following treatment of males with sGnRHa (t=6.25, P=0.001, N=6) but not saline (Fig. 3.1b).

A

Methanol extracted steroids

3 mV

5 sec

Pre-injection
sGnRHa

Post-injection
sGnRHa

B

Pre-Injection
Post-Injection

Normalized EOG response

*

6   6   3   3

sGnRHa   Saline

Methanol extracted steroids
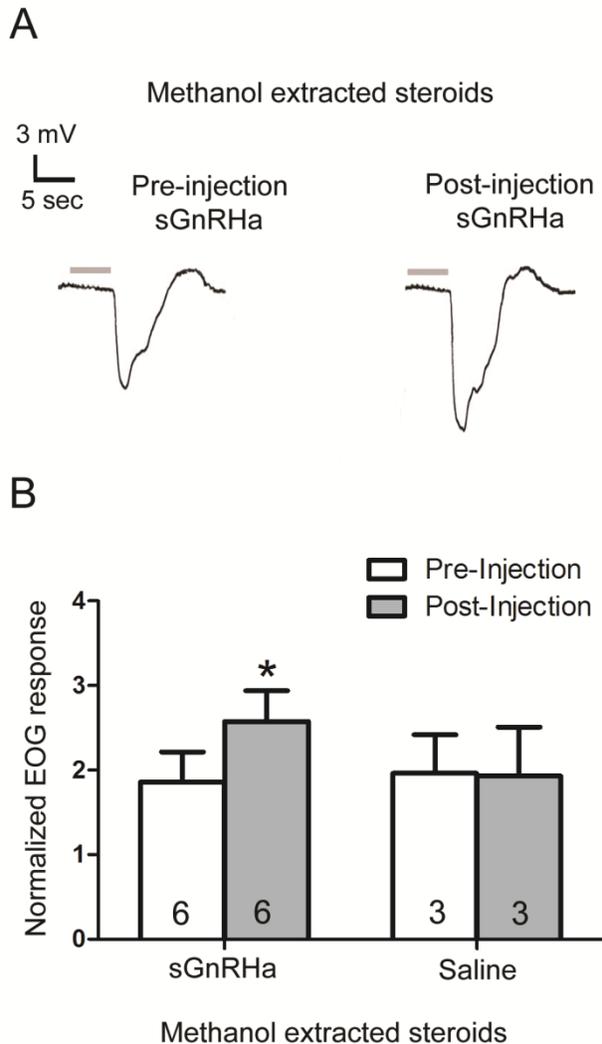
Fig. 3.1. EOG responses to methanol-extracted conditioned water containing steroids, from reproductive males (RM) before and after injection with either sGnRHa or saline. (A) typical EOG trace from female round gobies to pre-injection and post-injection (with sGnRHa) methanol extracts from an RM round goby. (B) female round gobies show an increased EOG response to the post-injection methanol extract of conditioned water when the RM goby has been treated with sGnRHa, but not saline.

3.4 Discussion

In order to synchronize spawning between the sexes, many fish species have evolved the use of hormonal products as pheromones, as these signals are indicative of the reproductive status of the sender. In goldfish – who have the best characterized reproductive system involving identified pheromones – females experience a surge in gonadotropin II in response to environmental factors, which in turn induces the females to release 17, 20β-P (Stacey et al.,

1989). Release of 17, 20β-P in turn stimulates a gonadotropin increase in the males (Stacey et al., 1989), and leads to the initiation of reproductive behavior (DeFraipont and Sorensen, 1993). Thus, GnRH is the necessary first step leading to spawning in both males and females, at least in this model species. Treating male gobies with sGnRHa causes an increased olfactory response from femals to male conditioned water, and it was previously shown that sGnRHa increases the release rate of putative pheromones (Katare et al., 2011). While I have not investigated the biological consequences of this increased olfactory response in this study, behavioural responses are underway, and it is highly plausible that exposure to these extracts could cause endocrinological effects as well. In goldfish, the reproductive system is reciprocal, with the males and the females stimulating each other via the release of different compounds into the water (reviewed in Stacey et al., 2003). I provide the first evidence for a similar situation in the round goby. I show that treating males with sGnRHa leads to a subsequent increase in female response to odours from these males. While Belanger et al. (2004) showed an increased EOG response from reproductive females but not non-reproductive females to reproductive male waters, this dataset indicates that the products released by sGnRHa treated males affect even non-reproductive females. We show that females may be attuned to – and subsequently stimulated by – endocrine changes in the male through release of chemical signals, providing additional evidence for the use of sex pheromones by the round goby.

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Chapter 4:

Female round gobies (*Neogobius melanostomus*) detect and discriminate between steroids released by male round gobies.

4.1 Introduction

The round goby *Neogobius melanostomus* (Pallas) is a small benthic teleost fish, and a highly successful invasive species to the Laurentian Great Lakes. This percid arrived via ballast water from the Ponto-Caspian region (Jude et al., 1992) and managed to invade all of the Great Lakes within five years of its introduction (Charlebois et al., 2001). RMs establish nests, then attract females for spawning and aggressively guard the nests against predation. The development of a biological control method employing pheromones to limit the spread of this species would be highly useful, as pheromones have the advantage of focussing on the target species (Corkum, 2004).

While the teleost reproductive pheromone system has been most thoroughly investigated in the goldfish (*Carassius auratus*; reviewed by Stacey et al., 2003), one of the earliest observations of a gonadal steroid attracting conspecifics was made in the black goby (*Gobius jozo*) by Colombo et al. (1977; 1980), who observed that females were attracted to 3α-hydroxy-5β-androstan-17-one 3-glucosiduronate (etiocholanolone glucuronide; ETIO-g), an androgen found in the gonads of this species. Other studies have since investigated reproductive communication in other fishes of the order Perciformes, including tilapia, *Oreochromis mossambicus* (Miranda et al., 2005; Barata et al., 2008) and the peacock blenny, *Salaria pavo* (Serrano et al., 2008). However, though fishes have long been used in the study of olfaction, most of what is currently known pertaining to the olfactory sense of fishes is confined to a few representative species including: goldfish, (e.g. Rolen et al., 2003; Hansen et al., 2004; Hansen et al., 2005); zebrafish, *Danio rerio* (e.g. Michel et al., 2003); channel catfish, *Ictalurus punctatus* (e.g. Hansen et al., 2003; Hansen et al., 2005), and Atlantic salmon, *Salmo salar* (Lo et

al., 1993; 1994). These species have long been bred in the lab or aquaculture facilities, and studies involving wild teleosts are rare.

In Ostariophysan fishes such as goldfish and the channel catfish, the cyclic adenosine monophosphate (cAMP) second messenger system mediates olfactory responses to amino acids and bile salts (Hansen et al., 2003; Rolen et al., 2003), and also to steroids in goldfish (Sorensen and Sato, 2005). This olfactory transduction cascade has long-been known in mammals as well, and is associated with olfactory sensory neurons in the main olfactory epithelium (reviewed by Ma, 2007). A transduction cascade utilizing phospholipase C (PLC)-stimulated inositol-1,4,5-trisphosphate (IP$_3$) was first identified in channel catfish (Restrepo et al., 1990), and has since been demonstrated to transduce amino acid odors in catfish (Hansen et al., 2003), as well as goldfish (Rolen et al., 2003) and zebrafish (Michel et al., 2003). This second messenger cascade is seen in the olfactory sensory neurons of the mammalian vomeronasal organ. Interestingly, Lo and colleagues (1993; 1994) found that in Atlantic salmon both amino acids and taurocholic acid (a bile salt) stimulate the production of PLC but not adenylyl cyclase – the only report of a fish that does not use cAMP to transduce amino acid and bile salt odorants. These studies provide strong evidence for roles for both cAMP and IP$_3$ in fish olfactory transduction. What is unclear, however, is whether more diverged fishes (such as the round goby) follow the trends seen in goldfish, zebrafish and catfish, with cAMP being the only second messenger seen to transduce bile salt odours, for example.

The peripheral olfactory organ of the round goby is composed of a single lamella covered in olfactory epithelium (Belanger et al., 2003), which is strikingly different from the multi-lamellar olfactory rosette seen in cyprinids and salmonids (Hansen and Zielinski, 2005). However, as in other teleost fishes, the olfactory epithelium of the round goby contains ciliated

olfactory sensory neurons expressing the $G_{\alpha olf}$, the GTP binding protein associated with sensory transduction via cAMP, and microvillous and crypt olfactory sensory neurons expressing $G_{\alpha o}$, which is associated with the phospholipase C/inositol triphosphate (IP$_3$) transduction pathway (Belanger et al., 2003). Recordings of summed generator potentials from the olfactory epithelium (electro-olfactogram, EOG) have revealed that round gobies detect a number of free and substituted 18- 19- and 21-carbon steroids representing putative pheromones (Murphy et al., 2001). The novel 5β-reduced steroids 11-oxo-etiocholanolone (11-O-ETIO) and a sulphated conjugate, 11-O-ETIO-3-s (Fig 4.1) are produced in the testes and seminal vesicle of RM round gobies (Arbuckle et al., 2004; Jasra et al., 2007), and released into the water (Katare et al., 2011), where these may be detected by females and possibly function as reproductive females. These steroids are considered novel as they have not been observed in any other fish species yet.
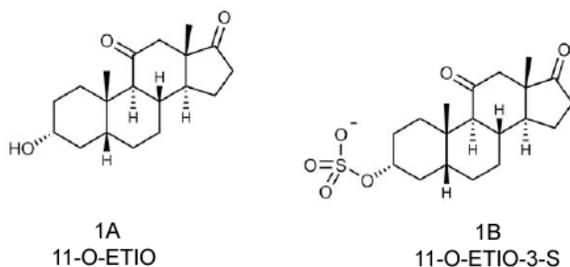
Fig 4.1: Chemical structures of 11-O-ETIO and 11-O-ETIO-3-s. Parts of this figure were originally published in Katare et al., 2011. Biol. Reprod. 84(2).

In this study I investigated the olfactory properties of the putative pheromones, 11-O-ETIO and 11-O-ETIO-3-s. I used electrophysiological methods to determine whether female round gobies could detect these compounds, and if so, what receptor mechanisms might be involved. I also investigated the odor potency of 11-O-ETIO and 11-O-ETIO-3-s on the goldfish (a representative of Cyprinid fish), to see if these putative round goby pheromones show signs of species specificity.

4.2 Materials and Methods

4.2.1 Experimental animals

Male and female round gobies (average weight: 11.9g; length: 9.5 cm) were angled or seined from the Detroit River and Lake Erie. Fish of both sexes were housed in the animal quarters, Department of Biological Sciences, at the University of Windsor in 400 L flow-through tanks or 75 L static tanks with charcoal filters, provided with dechlorinated municipal tap water between $16 - 26^{o}C$ (ambient temperatures). Fish were maintained on a 16 hour light: 8 hour dark photoperiod and fed daily with commercial tropical flake food (Aquatic Eco-Systems, Apopka, FL). Experiments were conducted using male and female round gobies between May and September in the years 2007-2010. Males and females were differentiated according to the shape of the urogenital papilla. Male round gobies have a long, pointed papilla while females have a short, wide two-lobed papilla (Bélanger et al., 2004). Since no differences in EOG responses (to amino acids or steroids) between male and female gobies have been observed (Murphy et al., 2001; Laframboise, unpublished), we used both sexes in these studies. Non-reproductive females (NRF) were utilized for the dose-response and cross-adaptation studies, and non-RM (NRM) round gobies for the second messenger studies.

Goldfish were also tested for EOG responses to the released round goby steroids. These were purchased from a local pet store (Profish, Windsor, ON) and held in aquaria with re-circulating filters under the same conditions as the round gobies. All animal collection, housing and experimental procedures were in accordance with the guidelines of the Canadian Council on Animal Care.

4.2.2 Electro-olfactogram recording

The electro-olfactogram (EOG) recording protocol was modified from that previously described (Murphy et al., 2001; Bélanger et al., 2004). Round gobies were anaesthetized via immersion in tricaine methanesulfonate (MS-222; Finquel®, Argent Chemical Laboratories, Redmond, WA, USA; 100 mg/l) and immobilized with an intra-muscular injection of gallamine triethiodide (Flaxedil, Sigma-Aldrich, Oakville, ON, Canada; 150 mg/kg). The test fish was then wrapped in a paper towel, placed in the recording chamber and a tube was inserted into the mouth providing dechlorinated water (with 20 mg/l MS-222) to the gills. The EOG was recorded differentially using thin wall glass capillaries (1.0 mm OD, 0.75 mm ID #TW100-4; WPI, Sarasota, FL, USA) pulled to a tip diameter of 80-100 µm, filled with 8% gelatine in 0.9% saline, and bridged to Ag/AgCl pellets with 3M KCl. Dechlorinated water flowed over the olfactory epithelium through an odor delivery tube positioned over the posterior naris. The reference electrode was placed lightly on the skin, near the anterior naris and the recording electrode was placed in the anterior naris and onto olfactory epithelium containing microvillous, ciliated and crypt olfactory sensory neurons (Belanger et al., 2003). The recording electrode was placed in the same general position with every recording, but the precise location of the electrode tip was not visible during recording and therefore was not necessarily entirely consistent. The electrical signal was amplified (model 7P122P, Grass Technologies, West Warwick, RI, USA), digitized (PowerLab model 4/30; ADInsturments, Colorado Springs, CO, USA) and displayed on a computer running Chart 5 software (AD Instruments, Colorado Springs, CO, USA). Switching from background water to stimulus odor was accomplished using a solenoid-driven mechanism, eliminating any possible mechanical stimulation. The EOG responses were measured (in millivolts, mV) as the amplitude of the negative deflection recorded in response to odorous

stimulation. Responses to water blanks were rare but when these occurred, the background response was subtracted from odor responses. There were no EOG responses to the carrier solutions containing methanol (0.0001% methanol for 10^{-8} M steroids) or DMSO (0.01% DMSO for the pharmacological inhibitor U73122).

4.2.3 Preparation of odors and pharmacological agents

Synthetic 11-O-ETIO (A3460-000), 11-O-ETIO-3-s (A3500-000), ETIO (A3610-000), and estrone (E2300-000) were purchased from Steraloids (Newport, RI, USA), dissolved in 100% methanol to make 10^{-2} M stock solutions and stored at -20°C, and on the day of testing the stimulatory solutions were prepared by serial dilution in dechlorinated water. The pharmacological agents used for investigating olfactory transduction, U-73122 (used to block IP$_3$ production; Rolen et al., 2003) and SQ 22,536 (used to block cAMP production; Sorensen and Sato, 2005), were purchased from Sigma-Aldrich (Oakville, ON, Canada; #U6756 and #S153) and Calbiochem (San Francisco, CA, USA; #662035 and #568500), diluted according to vendor instructions and stored as aliquots of 10^{-2} M stocks. The SQ 22-536 was prepared in double distilled water (used for its purity), aliquoted and frozen at -20°C until used, the U-73122 was dissolved in 100% DMSO, aliquoted and stored at -20°C. On the day of the EOG testing, fresh test aliquots were diluted in the same dechlorinated water as the background water that was perfused through the nares of the experimental fish. The 10^{-2} M stock solutions of the standards L-alanine and taurocholic acid (TCA) were made up fresh each day in this dechlorinated water and diluted serially to their final EOG testing concentrations of 10^{-5} M and 10^{-6} M, respectively. Amino acids and TCA were purchased from Sigma-Aldrich (Oakville, ON, Canada, #A7627, #T4009).

4.2.4 General EOG recording protocol and dose-response experiments

For all experiments, the EOG recordings conformed to methods previously described (Murphy et al., 2001; Rolen et al., 2003; Belanger et al., 2004). We used 5 second odor deliveries which typically resulted in 7-8 drops of odor being delivered to the epithelium, as well as shorter (1 second) deliveries, resulting in 2 drops being delivered, for the cross-adaptation experiments. Because of the nature of odor delivery systems for EOG recording, some researchers choose longer stimulus times in excess of 5 seconds (e.g. Miranda et al., 2005), while others use shorter durations of less than 5 seconds (e.g. Murphy et al., 2001). We did not see a difference in the magnitude of the EOG response with the shorter odor delivery time (i.e. with fewer drops of odor being delivered). At least 2 minutes were allowed between applications to avoid adaptation. The amino acid standard, 10^{-5} M L-alanine was delivered at the beginning and the end of the run, as well as after approximately every 10th odor delivery, to monitor the integrity of the recording. For dose-response testing, odors were typically given in ascending order from the lowest to highest concentration and were delivered at least three times per fish. To determine response thresholds, responses to odors (measured as mV) at each concentration were compared to responses to dechlorinated water using Student's t tests or Wilcoxin signed rank tests.

I also tested EOG responses to the putative round goby pheromones (11-O-ETIO and 11-O-ETIO-3-s) on a phylogenetically distant fish species, the goldfish. We followed the same EOG recording protocol as with the goby, with a few modifications. The flap of skin covering the goldfish naris was completely removed, exposing the olfactory rosette, and the recording electrode was placed between two lamellae, near the raphe, as is typical for recording goldfish EOG (e.g. Rolen et al., 2003). These preparations responded as previously described, to 10^{-4} M

L-serine (e.g. Hubbard et al., 2003), and to the steroid solution 10^{-8} M 17α,20β-dihydroxy-4-pregnen-3-one, 17α,20β-P (e.g. Sorensen et al., 1995).

4.2.5 Olfactory cross-adaptation

The olfactory cross-adaptation method compares the EOG response to an odor before and during adaptation to an adapting odorant. This test works under the assumption that if an odour receptor mechanism is separate from a compound that is mediating an adapting EOG response, its EOG response is unaffected by the adaptation (Caprio and Byrd, 1984; Sorensen et al., 1995; Sveinsson and Hara, 1990). Our cross-adaptation protocol followed that of Murphy et al. (2001). Firstly, to ensure that responses to steroid odors weren't naturally being reduced over time, we performed a sequential exposure experiment. For this, 10^{-8} M steroids (11-O-ETIO, 11-O-ETIO-3-s, and ETIO) were delivered in sequence. After the test fish was left for 30 minutes (to more closely approximate the total recording time of a serious of cross-adaptation experiments) with background water perfusing over the olfactory epithelium, these steroids were tested a second time in the same sequence. I evaluated whether previous exposure to each steroid odor reduced the response to the second exposure to that odor, by comparing the response of the second odor delivery (mV) to the response of the first delivery. The second response was expressed as percent initial response (% IR) – a percentage of the response to the first odor delivery.

The second part of the protocol comprised of the cross-adaptation experiment (Murphy et al., 2001). Firstly, 10^{-7} M (1 L) of the adapting compound (ETIO, 11-oxo-ETIO or 11-oxo-ETIO-3-S) was prepared, and was utilized both for adaptation and for diluting the odors tested for cross-adaptation. Once the test fish was found to be responding stably to the standard 10^{-5} M L-alanine (i.e. after 3 consecutive exposures to L-alanine), cross-adaptation testing began. The test steroid was delivered to the olfactory epithelium for 1 second at 10^{-8} M (pre adaptation),

followed by 2 minutes of continuous delivery of the adapting compound. During adaptation (after 2 minutes of continuous exposure to the adapting compound), the test steroid was delivered a second time (10^{-8} M), in the presence of the adapting compound, and the EOG response was recorded. The post adaptation recovery of the EOG response to the test steroid was measured following the flow of background water over the epithelium for approximately 5 minutes. This procedure was repeated for every combination of steroid (ETIO, 11-oxo-ETIO, 11-oxo-ETIO-3-S) as adapting and test compound. The EOG responses during adaptation were expressed as percentages of pre-adapted responses (as in the sequential exposure experiment). Although the data were expressed as the percent of the initial response (% IR) for statistical testing, all data (with one exception) passed tests of normality and equal variance, thus paired t-tests were used to determine which, if any steroid responses were reduced following adaptation. In the one case where the % IR data failed the test of equal variance (ETIO adapted to ETIO), a Mann-Whitney non-parametric test was performed. Data for all experiments were analyzed using SigmaStat 3.5 (Systat Software, Chicago, IL, USA).

4.2.6 Investigation of second messengers

I investigated the role of the second messengers cAMP and IP$_3$ in round goby olfactory responses by testing the effect of antagonists previously used in telesot EOG preparations (e.g. Rolen et al., 2003; Sorensen and Sato, 2005). The following representative odors were tested: L-alanine (amino acid), taurocholic acid (TCA, bile acid), 11-O-ETIO (a novel androgen released by round gobies, Katare et al., 2011) and estrone (an estrogen odor detected by the round goby; Murphy et al., 2001; Belanger et al., 2006). Odors were restricted to these representatives in order to simplify the testing procedure. Adenylyl cyclase activity was inhibited by perfusing the surface of the olfactory epithelium with 10^{-4} M SQ 22,536 for at least 2 minutes (Chen et al.,

2000; Sorensen and Sato, 2005). The PLC inhibitor U73122 (10^{-6} M) was delivered for 10 minutes, in accordance with previous studies (Jin et al., 1994; Hansen et al., 2003; Michel et al., 2003; Rolen et al., 2003). Suppliers of SQ 22,536 list IC_{50} values of between 1.4 x 10^{-6} M to 80 x 10^{-6} M depending on the preparation, and there is no specific IC_{50} value for this drug in olfactory sensory neurons, although it has been used in this way before. The concentration used here (10^{-4} M) is obviously within this range, and is in line with previous literature (Rolen et al., 2003). Similarly, vendors report a range of IC50 values for U73122 depending on the cell type or preparation, and our concentration was based on previous studies.

Each trial took place in three stages: first, (designated as "pre") dechlorinated background water was run over the olfactory epithelium for 10 minutes and EOG responses to odors were tested; second, (designated as "during") the chosen inhibitor was perfused over the naris for a set period of time (2 minutes for SQ 22-536 and 10 minutes for U73122) and the odor responses were recorded again in the presence of the inhibitor; lastly (designated "post") the recovery of the EOG odor responses was tested following the removal of the pharmacological agent by 10 minutes of background water flow over the olfactory epithelium. Trials were included in the data analysis only if the post-treatment (recovery) value was at least 30% of the pre-treatment value. Pre and during measurements for each odor response under each inhibitor were compared using paired t-tests or Wilcoxen Signed Rank test (in one instance where the data were not normally distributed – alanine responses under SQ 22,536 treatment) to determine whether the inhibitor significantly reduced odor responses.

4.3 Results

4.3.1 Round goby EOG responses to synthetic analogues of released steroids.

Table 4.1 summarizes the results. I tested olfactory responses to the novel steroids 11-O-ETIO and 11-O-ETIO-3-s (Fig 4.1), which are synthesized by the gonads of the round goby (Arbuckle et al., 2005; Jasra et al., 2007) and released into the water (Katare et al., 2011). These steroids have not been reported in any other fish species. Female round gobies showed characteristic olfactory epithelial responses to the synthetic steroids in a dose-dependent manner, while goldfish failed to detect either 11-O-ETIO or 11-O-3-S at any of the three concentrations tested (Fig. 4.2A, B). The EOG responses to 10^{-8} M and 10^{-9} M 11-O-ETIO were 1.43 ± 0.28 mV and 0.927 ± 0.08 mV respectively; both were significantly different than background responses (0.13 ± 0.04 mV; P<0.01). The response profile to 11-O-ETIO-3-s was similar to free 11-O-ETIO (Fig 4.2B). The EOG responses from these NRF round gobies were significantly different from background responses (0.15 ± 0.04 mV) at 10^{-8} M 11-O-ETIO-3-s (1.53 ± 0.25 mV; P<0.05) and 10^{-9} M 11-O-ETIO-3-s (0.93 ± 0.15 mV; P<0.01). These findings indicate that 11-oxo-ETIO and 11-oxo-ETIO-3-S are potent odors to the round goby, but not to the goldfish.
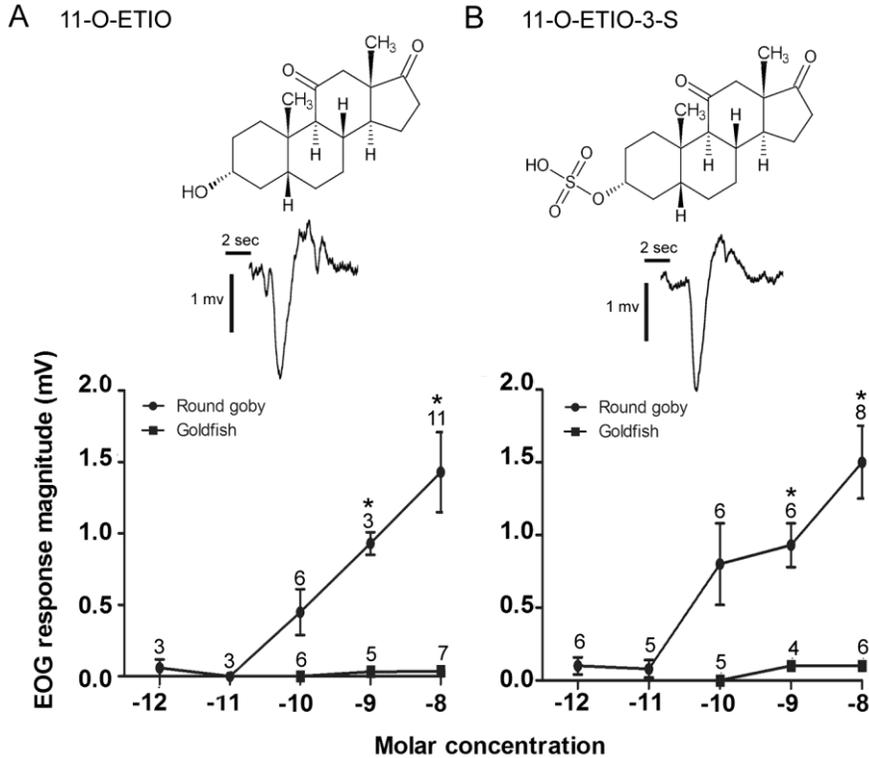
Fig. 4.2. Female round goby and goldfish electro-olfactogram (EOG) responses to steroids 11-O-ETIO and 11-O-ETIO-3-s (3α-hydroxy-5β-androstane-11,17-dione 3-sulfate) released by male round gobies. (A) The round gobies respond to 11-O-ETIO in a typical dose-dependant manner. The structure of 11-O-ETIO is shown, as is a representative EOG tracing (10^{-8} M). Goldfish did not detect 11-O-ETIO . (B) The structure of 11-O-ETIO-3-s is shown, along with a representative trace of an EOG response. EOG responses of female round gobies to 11-O-ETIO-3-s are dose-dependant, but responses were not observed in goldfish. Asterisks indicate that responses are statistically different from responses to background water. The number of fish tested is indicated for each concentration.

4.3.2 Olfactory cross-adaptation

I investigated olfactory discrimination of 11-O-ETIO, 11-O-ETIO-3-s and ETIO by testing for olfactory cross-adaptation. The control sequential experiment tested for the effect of prior exposure to an odor on subsequent EOG responses to the particular odor. The three odors were not significantly reduced by prior exposure 30 minutes earlier (Fig. 4.3A; $P>0.05$). Cross-adaptation was investigated by observing if a steroid elicited an EOG response during adaptation to a second steroid. When 11-O-ETIO was used as the adapting compound, olfactory responses

to the same steroid were significantly reduced to 35.2% ($\pm$ 3.2%) of their initial response magnitude (P<0.05), while responses to 11-O-ETIO-3-s (88.9 $\pm$ 15.1%) and ETIO (115.1 $\pm$ 40.2%) were not significantly reduced (Fig. 4.3B; P>0.05). When the olfactory epithelium was adapted to 11-O-ETIO-3-s (Fig. 4.3C), responses to 11-O-ETIO-3-s (23.3 $\pm$ 8.6%) were significantly reduced (P<0.001), while responses to ETIO and 11-O-ETIO were not significantly reduced. Similarly, continuous exposure to ETIO only had an effect on ETIO-evoked responses, reducing them to 36 $\pm$ 7.7% (Fig. 4.3D; P<0.01). These findings show that cross adaptation between ETIO, 11-O-ETIO and 11-O-ETIO-S did not occur, and indicate that all three steroids activate different receptor mechanisms.
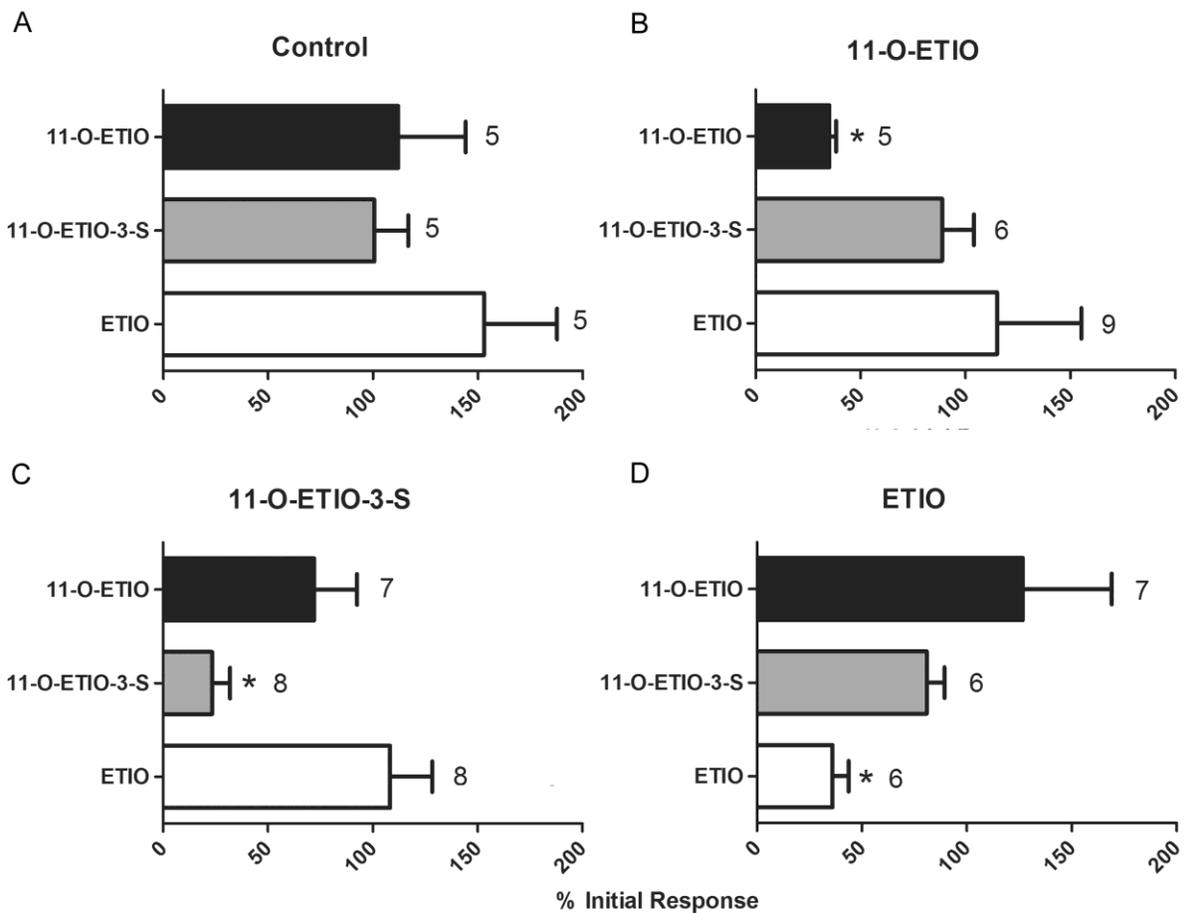
Fig. 4.3. Olfactory cross-adaptation. Percent initial EOG responses (% IR) to 10^{-8} M 11-O-ETIO, 11-O-ETIO-3-s and ETIO. The adapting compound (10^{-7} M) is shown on the top. (A) %

IR during the control sequential exposure experiment. None of the steroids were significantly affected by 30 minute prior exposure to the odorant, though ETIO had a tendency to increase upon second odor delivery. (B) During adaptation to 10^{-7} M 11-O-ETIO, EOG responses to 11-O-ETIO were significantly reduced, while the other steroids were unaffected. (C) While 10^{-7} M 11-O-ETIO-3-s was the adapting compound, only the responses to 11-O-ETIO-3-s were significantly reduced. (D) Adaptation to 10^{-7} M ETIO reduced EOG responses to ETIO, while leaving the other steroid responses largely unaffected. The asterisks indicate that the changes during adaptation were significantly different from the change during the control experiment (Fig. 2B).

4.3.3 Blocking of adenylyl cyclase/cAMP by SQ 22,536

I recorded EOG responses to odors representing four different odor classes: amino acids (alanine), bile acids (TCA), androgens (11-O-ETIO, a putative pheromone specific to the round goby), and estrogens (estrone, an estrogen detectable by the round goby, Murphy et al. 2001), and compared EOG responses before and during treatment with the adenylyl cyclase antagonist SQ 22,536. Two minutes of perfusing the adenylyl cyclase blocker SQ 22,536 (10^{-6} M) over the olfactory epithelium significantly reduced olfactory response amplitudes to 10^{-6} M TCA ($P<0.01$), 10^{-8} M 11-O-ETIO ($P<0.05$) and 10^{-8} M estrone ($P<0.05$) compared to the pre- SQ 22,536 amplitudes (Fig. 4.3A). However, responses to 10^{-5} M alanine were not significantly reduced by pre-treatment with SQ 22,536 ($P>0.05$). These findings demonstrate that cAMP is used in the transduction of bile acids and steroids, but not amino acids.

4.3.4 Blocking of PLC/IP3 by U73122

In order to investigate the role of PLC-activated IP$_3$ in olfactory signal transduction, I treated the olfactory epithelium with 10^{-6} M U73122 for 10 minutes. This treatment significantly reduced olfactory responses to 10^{-5} M alanine ($P<0.01$), 10^{-8} M 11-O-ETIO ($P<0.05$), and 10^{-8} M estrone ($P<0.05$), indicating that amino acids, and steroid odors utilize the PLC/IP$_3$ signalling cascade; however, TCA-evoked responses were not significantly reduced following U73122 treatment (Fig. 4.4B).
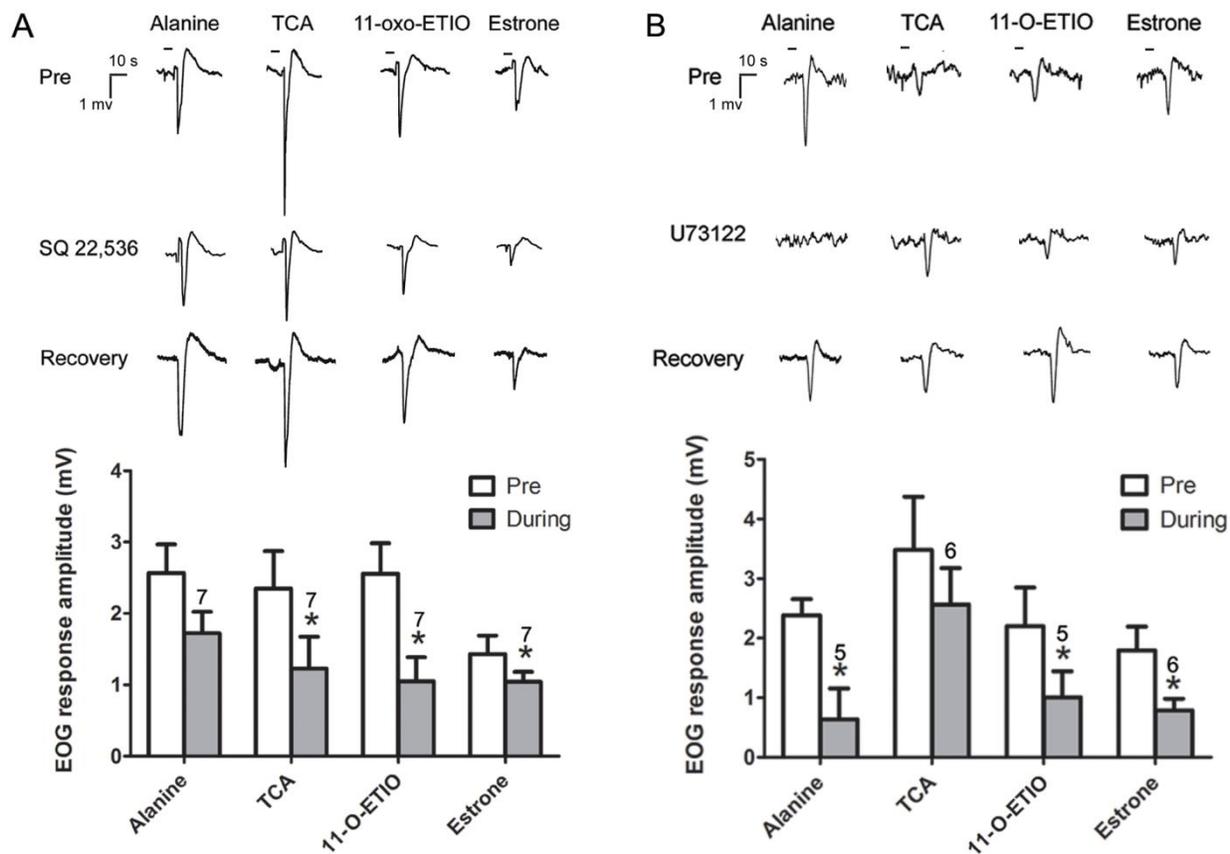
Fig. 4.4. EOG responses to different odor classes during inhibition via antagonists of cAMP and IP$_3$. Traces show EOG responses before, during and after treatment with U73122. The horizontal bars above each "pre" tracing indicates the 5 second odor delivery. (A) Representative traces show EOG responses for all odors before and during exposure to SQ 22,536 and following a 10 minute recovery. Exposure of the olfactory epithelium to the cAMP antagonist SQ 22,536 reduced responses to taurocholic acid (TCA), 11-O-ETIO and estrone, but not alanine. (B) Exposure to the IP$_3$ antagonist U73122 caused a reduction in EOG responses to alanine, 11-O-ETIO and estrone, but not TCA.

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Table 4.1: Summary of results for these EOG experiments. Where appropriate, results from Murphy et al., 2001 are included. These data are indicated by asterisks (*).

Odour	EOG Threshold	Receptor	Second Messenger
11-O-ETIO	10^{-9} M	Some specificity for 11-O-ETIO	cAMP and IP$_3$
11-O-ETIO-3-s	10^{-9} M	Some specificity for 11-O-ETIO-3-s	Not tested
ETIO	10^{-9} M *	Non-specific. Detects ETIO-g, androstenedione and others*	Not tested
Estrone	10^{-9} M *	Non-specific. Detects estradiol*	cAMP and IP$_3$
L-Alanine	Not tested	Not tested	IP$_3$
TCA	Not tested	Not tested	cAMP

4.4 Discussion

These findings provide supporting evidence of pheromone functionality for 11-O-ETIO and 11-O-ETIO-S, two androgens released by reproductive male (RM) round gobies (Katare et al., 2011). Nanomolar 11-O-ETIO and 11-O-ETIO-3-s elicit field potential responses from the olfactory epithelium of conspecifics, but not from goldfish, and the round gobies are able to discriminate these steroids at the sensory level. Additionally, olfactory transduction occurs via previously characterized second messengers

Pheromone communication during reproduction was seen in the black goby over thirty years ago, when Colombo et al. (1977; 1980) reported that reproductive females are attracted to ETIO-G, a substituted steroid produced in the gonads of RMs. Since RM round gobies build and maintain nests on the lake bottom, within which many females deposit eggs (Wicket and Corkum, 1998), pheromone communication is postulated for this species as well. Investigation of the chemical identity of the round goby reproductive pheromones began with Murphy et al. (2001), who demonstrated olfactory epithelial field potential responses to several synthetic

steroids including ETIO, ETIO-G and other androgens (C_{19} steroids) with a 5β,3α-reduced configuration. However, 11-O-ETIO is the dominant product when the testes and seminal vesicles of RMs convert androstenedione to 5β,3α-reduced steroids *in vitro* (Arbuckle et al., 2005; Jasra et al., 2007). Bélanger (2004) found that 11-O-ETIO is detected by round gobies as an odour, and most recently, Katare et al., (2010) demonstrated that free and substituted 11-O-ETIO (including 11-O-ETIO-3-s) are released by RMs. Therefore, water that previously held RMs and is attractive to reproductive females (Gammon et al., 2005), and extracts of RM water that evokes strong EOG responses in reproductive females (Bélanger et al., 2004) likely contain 11-O-ETIO and 11-O-ETIO-3-s. Behavioural responses to mixtures of synthetic steroids that include 11-O-ETIO and 11-O-ETIO-3-s (Corkum et al., 2008) and to isolates with these compounds, collected from water that held RMs (Kereliuk, 2009) also support pheromonal function of these steroids.

The EOG cross adaptation experiments in this study show that 11-O-ETIO and 11-O-ETIO-S are discriminated at the sensory level. It has long been suspected that the pheromone that attracts the reproductive females is likely to be a substituted rather than a free steroid. When RM water extracts are separated on reverse-phase high-performance liquid chromatography (HPLC), reproductive females show significantly higher field potential responses from the olfactory epithelium (EOG) to fractions that correspond to the elution positions of substituted rather than free steroids (Bélanger et al., 2004). Behavioural experiments also show that solutions containing 11-O-ETIO or 11-O-ETIO-3-s evoke different behavioural responses (Corkum et al., 2008; Kereliuk, 2009). While the unsubstituted steroids are likely excreted continuously from the gills (Vermeirssen et al., 1996) thus creating an uninterrupted plume, it is well known that urine is the main route of excretion for substituted steroids in fish (Scott and

Vermeirssen,1994). Meunier's (2009) observation that nesting RMs release more urine in the presence of females, and the discovery of 11-O-ETIO-3-s in the urine of RM round gobies (Katare et al., in press), also indicate that the urine (containing 11-O-ETIO-3-s) conveys specific chemical information to conspecific recipients.

Ligand competition studies using field potential recordings from the olfactory epithelium (cross-adaption) show that both substituted and free ETIO (ETIO, ETIO-G, 11β-ETIO-G) interact with the same receptor, and this one receptor also interacts with androstenedione (Murphy et al., 2001). On the other hand, 11-O-ETIO and 11-O-ETIO-3-s activate independent receptor mechanisms, an interesting distinction that may hint at the importance of these compounds as olfactory cues for the round goby. It may be that the ETIO receptor mechanism described by Murphy et al., (2001) represents a more generalized receptor responding to some androgens, conveying information to the female about the sex of the male. If 11-O-ETIO and 11-O-ETIO-3-s are indeed pheromones, they may convey more specific information about the reproductive status of the male. My results indicate that the round goby olfactory system discriminates between these odors, which could lead to different behavioral responses, as independent and specific olfactory receptor sites for pheromones appear to be important for initiating the appropriate behaviour (Poling et al. 2001, Sorensen et al., 1995). It is also possible that both steroids in a mixture could be necessary to elicit a reproductive response, as is the case in several species of moth (Linn et al., 1985).

There have been few olfactory cross-adaptation studies of fish pheromones. In the sea lamprey (*Petromyzon marinus*), separate compounds identified as migratory (Fine and Sorensen, 2008; Li et al., 1995) and reproductive (Siefkes and Li, 2004) pheromones activate different olfactory receptors. In the goldfish, the pheromones 17,20β-P and 17,20β- P-20-sulfate have

independent receptors (Sorensen et al., 1995), as well as different behavioral properties (Poling et al., 2001). The prostaglandin pheromones PGF2α and 15-oxo-PGF2α also activate different receptor mechanisms in goldfish (Sorensen et al., 1988). The sea lamprey, goldfish and round goby represent three different groups of fishes, but the possibility that pheromones have individual receptors is supported in all three species.

This is the first study of teleost fish that investigates the olfactory properties of synthetic analogues of steroids released by reproductive conspecifics. This strategy has been used for investigating sea lamprey pheromones (Li et al., 1995; Siefkes and Li, 2004). Most current research takes one of two approaches: testing a specific compound and for possible biological activity (e.g. behavior or EOG) without knowing if it is released by the conspecifics (e.g. Giaquinto and Hara, 2008), or testing isolates of conditioned water or urine on opposite-sex conspecifics (e.g. Barata et al., 2008; Serrano et al., 2008).

This is the third time that unique, released compounds have been proposed as pheromones in a perciform species, since Colombo identified ETIO-G as a pheromone in the black goby (1977; 1980), and Barata et al., (2008) tentatively identified a sterol-like compound released by male Mozambique tilapia (*Oreochromis mossambicus*) which the females may use to identify dominant males.

Although many fish species have been investigated for the possible use of pheromones, species specificity has not been well-investigated in fishes (Sorensen and Stacey, 1999). The round goby detects neither 17,20β-P nor prostaglandin $F_{2\alpha}$, both of which are important goldfish pheromones (Sorensen et al., 1988; Stacey et al., 1989) and are detected by other cyprinids, including zebrafish (e.g. Belanger et al., 2009). Indeed, overall it appears as though F-prostaglandins are more likely to have pheromonal actions in cyprinid fishes than in non-

cyprinids such as the goby (Kitamura et al., 1994). The cichlid fish *Astatotilapia burtoni* responds only to substituted steroids but not free steroids (Robinson et al., 1998). Bile salt odors – which may be pheromones in rainbow trout, *Oncorhynchus mykiss* (Giaquinto and Hara, 2008) – were shown to be equally potent odors in goldfish, Mozambique tilapia and European eel (*Anguilla anguilla*), indicating that bile salts may function as pheromones in a non-specific manner in many different fishes (Huertas et al., 2010). Although I show that the goldfish olfactory system does not respond to the steroids released by the round goby, it is far too early in this investigation to say that these compounds are specific to only the round goby; however, the specificity of the steroids is important if they are to be used as a biocontrol method.

My investigation of olfactory sensory transduction of three odor classes (bile acids, steroids and amino acids) reveals similarities as well as differences between this process in the round goby (an Acanthopterygiid perciform) and the more commonly studied Ostariophysan fishes: zebrafish (Ma and Michel, 1998; Michel, 1999), catfish (Hansen et al., 2003), and goldfish (Rolen et al., 2003); as well as salmonids (Lo et al., 1993; 1994). TCA responses are not affected by treatment with the IP_3 inhibitor, and though the power of this test was somewhat low, this result has been seen in other species and thus is likely to be correct despite the low power of the test. The reliance of bile acid (TCA) odor transduction solely on cAMP as a second messenger in the round goby is in agreement with studies of Ostariophysan fish (Ma and Michel, 1998; Michel, 1999; Hansen et al., 2003; Rolen et al., 2003). It appears that the odor reception of bile salts represents a conserved mechanism across those fishes thus examined – with a single exception; Lo et al., (1994) reported that TCA signal transduction occurs via IP_3 in Atlantic salmon. A wealth of evidence has shown that olfactory responses to bile salts are mediated by ciliated olfactory sensory neurons possessing receptors which are coupled to a G protein

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expressing the α subunit $G_{\alpha olf}$ and utilizing the adenylyl cyclase/cAMP cascade (Nikonov and Caprio, 2001; Sato and Suzuki, 2001; Hansen et al., 2003; Rolen et al., 2003; reviewed by Hansen and Zielinski, 2005). In the round goby, ciliated olfactory sensory neurons expressing $G_{\alpha olf}$ are distributed within the olfactory epithelium (Belanger et al., 2003); which leads us to suppose that TCA also binds onto receptors on ciliated OSNs in this species.

The use of second messengers in the transduction of amino acids shows diversity across taxa. In the round goby, EOG responses to the amino acid L-alanine are unaffected by the adenylyl cyclase inhibitor, yet decline during treatment with U73122, indicating that transduction of alanine uses the PLC/IP$_3$ pathway. In zebrafish, IP$_3$ singly transduces amino acid odors (Michel, 1999; Michel et al., 2003); however, both IP$_3$ and cyclic AMP cascades transduce amino acids in catfish (Hansen et al., 2003) and goldfish (Rolen et al., 2003). In these studies of catfish and goldfish amino acid mixtures were utilized for stimulating the olfactory epithelium; thus it is possible that different amino acids may use different second messengers. However, Ma and Michel (1998) tested several different types of amino acids separately and found that in the zebrafish, all amino acid responses are reduced by blocking IP$_3$ via ruthenium red or neomycin. It is possible that differences in methodology – namely, in the blocker used – may account for some differences in results, as the exact mechanisms of these antagonists are not always clearly defined. For example, Sorensen and Sato (2005) found no effect on goldfish responses to amino acids using MDL-12,3330A to inhibit cAMP production, while Rolen et al., (2003) observed a decline in amino acid-evoked EOG responses with SQ 22,536. In this study there was a non-significant decrease in the amino acid odour with SQ 22,536 treatment, but the power of the test was appropriate, lending confidence to the results. As TCA is detected by ciliated OSNs, studies show that amino acids odors are processed at least in part by microvillous olfactory sensory

neurons in salmonids and cyprinids (e.g. Hansen et al., 2003; Sato and Suzuki, 2001). Receptors on microvillous olfactory sensory neurons are coupled to the G protein subunit $G_{\alpha o}$ or $G_{\alpha q/11}$ (Hansen et al., 2003; Hansen et al., 2004). In the round goby, microvillous OSNs are immunolabelled for the $G_{\alpha o}$ subunit and, like $G_{\alpha olf}$, are distributed throughout the olfactory epithelium (Belanger et al., 2003). It is likely, then, that the round goby detects amino acid odors through receptors found on microvillous olfactory sensory neurons.

The results observed in this study point to unique properties for olfactory transduction of steroids in the round goby. Inhibitors of cAMP and IP_3 pathways suppress responses to 11-O-ETIO and to estrone; indicating that both second messenger pathways are used in the olfactory transduction of these steroids, which differ considerably, with respect to chemical structure and biological activity. Additionally there is no cross-adaptation between the estrogen and androgen steroids in the round goby (Murphy et al., 2001), indicating that these activate separate receptors. A different strategy is seen in goldfish, which uses cAMP, and not IP_3 for transducing a solution containing a mixture of the steroidal sex pheromones, 17α,20ß-P, 17α,20ß-P sulfate and androstenedione (Sorensen and Sato, 2005). 17α,20ß-P, 17α,20ß-P sulfate fails to elicit olfactory activity in the round goby (Murphy et al., 2001), and although androstenedione (a precursor of 11-O-ETIO) functions as an odorant to the round goby (Murphy et al., 2001), the second messenger(s) associated with androstenedione olfactory transduction in the round goby are not known. As steroids appear to use both second messenger cascades in the round goby, this indicates that steroid odors could be activating different receptor proteins on both types of olfactory sensory neurons. Despite the long-held convention that mammals detect pheromones through specific receptors located on olfactory sensory neurons of the vomeronasal organ, it is

now clear that the main olfactory epithelium also contains receptors capable of recognizing pheromonal odors (recently reviewed by Rodriguez and Boehm, 2009; Ma, 2007).

In conclusion, I present physiological evidence that the released steroids 11-O-ETIO and 11-O-ETIO-3-s are reproductive pheromones in the round goby, and that these steroids may be perceived as individual odors. While the round goby, like other teleosts transduces bile acid odors through a cAMP cascade; unexpectedly, the steroid odours may utilize both and cAMP and IP_3 cascades. Overall, the results of this study support the view of diversity of olfactory function in teleost fish – especially with respect to the identity and transduction of reproductive pheromones. Additionally, the round goby presents itself as a good study species for investigations of pheromones, given its ecological importance and phylogenetic positioning.

4.5 References

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Chapter 5:

Female round goby olfactory responses to male urine and synthetic analogues of steroids: 11-O-ETIO-17-s, 11-O-ETIO-3-g, 3α,17β-dihydroxy-5β-androstan-11-one, cortisol, and cortisone.

5.1 Introduction

The round goby (*Neogobius melanostomus*), native to the Ponto-Caspian region of Eurasia, is an invasive species in the Laurentian Great Lakes. This small, benthic teleost inhabits dark, turbid environments where vision most likely does not function as a means of communication over distances for this colonial breeding species. Reproductive male (RM) round gobies establish and guard nests and eggs (MacInnis and Corkum, 2000), and display prominent secondary sexual characteristic (Miller, 1984), while non-reproductive males (NRM) do not. In addition, male round gobies exhibit different male morphs. The large parental RMs – in addition to displaying secondary sexual characteristics – have gonads with large accessory glands (compared to the testes), while smaller sneaker males have large testes but small accessory glands (Marentette et al., 2009). It is hypothesized that these sneaker males may act as female mimics and have larger testes in order to release more sperm to compete with the parental RM for fertilizations, while the parental RMs have larger accessory glands for the production of pheromones (Marentette et al., 2009). It has been posited that the nesting RMs utilise novel steroidal pheromones as a means of attracting females. Females are attracted to – and show increased olfactory responses to – RM conditioned water (Bélanger et al., 2004; Gammon et al., 2004). Round gobies detect diverse 18- 19- and 21-carbon steroids (Murphy et al., 2001) and are attracted to blends of free and substituted steroids (Corkum et al., 2008; Kereliuk et al., 2009). Reproductive male round gobies synthesize (Arbuckle et al., 2005; Jasra et al., 2007) and release (Katare et al., 2011) the novel 5β-reduced androgen 11-oxo-etiocholanolone (11-O-ETIO; Fig 1A) in both free and substituted forms. The substituted forms of 11-O-ETIO vary based on the positioning of the sulfate or glucuronide group, and the release of four substituted forms have been observed (Fig 5.1; originally published in Katare et al., 2011): 11-O-ETIO-3-sulfate (11-O-

ETIO-3-s; Fig 5.1B), 11-O-ETIO-17-sulfate (11-O-ETIO-17-s; Fig 5.1E), 11-O-ETIO-3-glucuronide (11-O-ETIO-3-g; Fig 5.1C), and 11-O-ETIO-17-glucuronide (11-O-ETIO-17-g; Fig 5.1F; Katare et al., 2011). When urine is probed by ELISA, the main immunoreactive conjugate is 11-O-ETIO-17-s, though 11-O-ETIO-3-g is also present in RM urine following injection with gonadotropin releasing hormone (Katare et al., 2011). Electro-olfactogram (EOG) studies show that 11-O-ETIO and 11-O-ETIO-3-s are equally potent odourants (Chapter 4), but what of the other identified released compounds? Substituted 11-O-ETIO is released via the urine (Katare et al., 2011). Furthermore, nesting RMs release urine in pulses in the presence of reproductive females (but not non-reproductive females); this indicates that the RMs actively signal to the females by through urine release (Meunier 2009).

Urine is a main route of pheromone release in fishes. As a route of evacuating compounds from the body, urine contains many compounds, several of which may affect the EOG (such as proteins and ions). It is known that urine can signal reproductive status and attract opposite sex conspecifics in cyprinid fishes like goldfish, *Carassius auratus* (reviewed by Stacey et al., 2003), and salmonids such as masu salmon, *Oncorhynchus masou* (Yambe et al., 2006). Urine also signals social dominance of males in Mozambique tilapia, *Oreochromis mossambicus* (Barata et al., 2008). In Atlantic salmon (*Salmo salar*), female urine has been shown to have a priming effect on males, and may be used to synchronize reproduction (Waring et al., 1996). While it is known that pheromones are released via the urine, urine is also a route of release for many other compounds which may not act as pheromones and are merely being excreted from the body. Whether female round gobies show an overall increased olfactory response to RM urine is unknown, but similar results have been seen in other species. For example, in the

Mozambique tilapia, females had greater EOG responses to urine collected from dominant males compared to urine from subordinate males (Barata et al., 2008).

When RM round gobies were treated with salmon gonadotropin releasing hormone analogue (sGnRHa), females showed an increased olfactory response to extracts from male-conditioned water, but the responses to extracts did not increase when the RM gobies were injected with saline (Chapter 4). This conditioned water contains compounds from several release routes: urine, mucus from the gills and feces. In addition, given the stressful conditions of conditioned water collection and handling for treatment with sGnRHa, the males could be releasing cortisol, a glucocorticoid associated with stress, or cortisone, a metabolite of cortisol (Wysocki et al., 2006). It cannot be ruled out that the olfactory potency of extracts of male conditioned water seen in Chapter 4 could be due, at least in part, to the presence of cortisol or cortisone in the extracts, although it was shown that injection with sGnRHa did not increase the release rate of cortisol, and in this study, release of 11-O-ETIO was about 20x greater than cortisol release (Katare et al., 2011).

I olfactory responses recorded from female round gobies in response to synthetic analogues of steroids released by male round gobies (11-O-ETIO-17-s, 11-O-ETIO-3-g, 3α,17β-dihydroxy-5β-androstan-11-one), as well as to cortisol, and cortisone. Additionally, this chapter describes the responses of non-reproductive female round gobies to urine collected from reproductive and non-reproductive males following wild capture and without the influence of sGnRHa treatment.

5.2 Methods and Materials

5.2.1 Experimental animals

Male and female round gobies were angled or seined from the Detroit River in Windsor, Ontario during June – August, 2008 and 2009, and transported to the University of Windsor animal care facilities, where they were housed either in flow through tanks supplied with dechlorinated municipal tap water or in aquaria with recirculating charcoal filters and dechlorinated municipal tap water. Water temperatures were between $18 - 26^{o}C$ (i.e. ambient), and the fish were kept on a 16:8 hr photoperiod. Round gobies were fed once daily with commercial fish flakes (Aquatic Eco-systems, Apopka, FL, USA). Males and females were sexed based on the shape of their urogenital papillae (Miller, 1984) and housed separately. Reproductive males (RMs) were differentiated from non-reproductive males (NRMs) based on their display of secondary sexual characteristics such as dark nuptial colouration, thick slime coat and puffy cheeks (MacInnis and Corkum, 2000). Fish may have been held for up to one month before physiological testing, but most individuals were used within 2 weeks. All procedures were in accordance with CCAC guidelines.

5.2.2 Steroid and odour preparation

Synthetic steroids were purchased from Steraloids (Newport, RI, USA): 11-O-ETIO-3-g (#A3470-000), 11-O-ETIO-17-s (#A3232-000), cortisol (#Q3880-000) and cortisone (#Q2500-000). The steroid 3α,17β- dihydroxy-5β-androstan-11-one (Fig 5.1D) was generated by Dr. Yogesh Katare and Dr. Alexander Scott by acid solvolysis of 11-O-ETIO-17-s as follows: 1 mg/ml of 11-O-ETIO-3-s dissolved in methanol was evaporated in the speedvac. The resulting residue was in distilled water and trifluoroacetic acid in ethyl acetate (1.4:100 v:v). This solution was incubated at $45^{o}C$ for 18 h, followed by evaporation of the solvent under nitrogen. The

residue was reconstituted in dechlorinated background water and diluted to the appropriate concentration for testing (10^{-8} M). The standard odour L-alanine was purchased from Sigma-Aldrich (Oakville, ON, Canada). Steroids were prepared initially as 1 mg/ml stock solutions in 100% methanol and stored at -20°C. On the day of testing, steroids were dissolved in dechlorinated background water to make 10^{-4} M solutions, and then serially diluted in dechlorinated water to the testing concentrations, the highest of which was 10^{-8} M. This concentration of steroid is typically the most potent tested in EOG studies (e.g. Murphy et al., 2001). Stock solutions of 10^{-2} M L-alanine were made up fresh daily and diluted serially in dechlorinated water to the testing concentration of 10^{-5} M.

Steroid stock solutions used for EOG testing were analyzed via mass spectrometry to establish that they were free of contamination (Appendix C). The spectrum for the stock solution for 11-O-ETIO-17-S showed only one peak, while the spectrum for the 11-O-ETIO-3-G show two peaks, indicating some impurity. This was confirmed through thin layer chromatography by Steraloids. All mass spectrometry was performed by Miss Manika Gupta
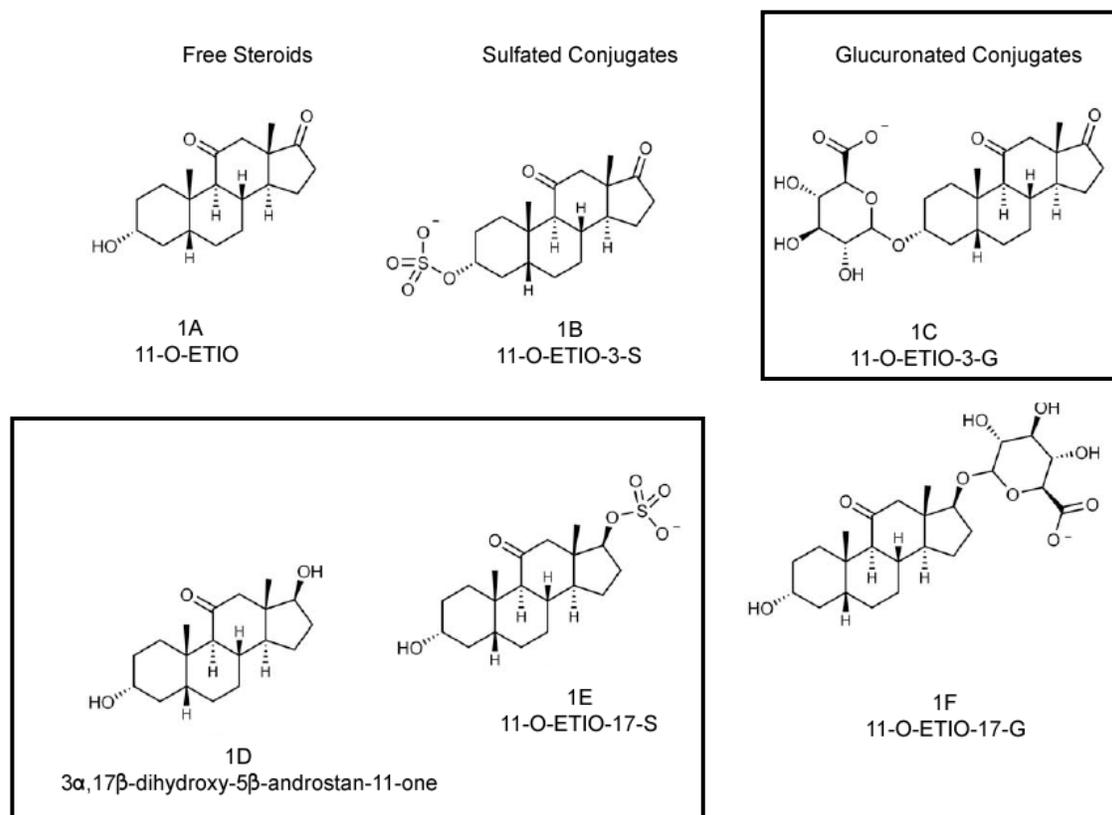
Fig 5.1: Chemical structures of steroids relevant to this thesis. Steroids specifically tested in this chapter are boxed. Parts of this figure were originally published in Katare et al., 2011. Biol. Reprod. 84(2). The release of all of these steroids was confirmed except for compound 1D which is a reactive intermediary that is probably not released.

5.2.3 Urine collection and preparation

EOG recordings were made in response to urine samples collected from RM round gobies in the field; kindly provided by Stan Yavno. Following capture, the RMs were lightly anesthetized with clove oil and the urogenital papilla was tied tightly with dental floss. The RMs were then isolated in buckets containing aerated river water for 4 hours, allowing for urine to collect in the bladder. After 4 hours the males were given an overdose of clove oil and urine was extracted by puncturing the bladder though the skin with a 25 gauge needle. These urine samples were stored at -20°C until use (Yavno and Corkum, 2010).

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Thawed urine samples were diluted for EOG testing as follows: first, the amount of urine used for EOG testing was standardized by using 0.1 of the total volume of urine collected from a male goby. For example, for urine # 18, we saved 8.8 µl for EOG testing, meaning that 88 µl of urine was collected from this fish (Appendix D). A stock urine solution was prepared by diluting 10x in dechlorinated water. For example, for urine #18, the 8.8 µl was diluted in 79.2 µl of water (the volume of the urine stock solution was 88 µl). The stock solutions were then diluted serially in dechlorinated water 100x, for EOG testing. These dilutions were designated 100x, 10,000x, 1,000,000x and 100,000,000x. The amount of immunoreactive substituted 11-O-ETIO in each urine sample was measured by ELISA (Dr. Yogesh Katare, U. of Windsor; Appendix D). 11-O-ETIO17-S accounts for the most immunoreactivity in the urine (Katare et al., 2011). Thus, the *estimated* molarity of the urine samples was calculated based on the molecular weight of 11-O-ETIO-17-s, which was 385 (Katare et al., 2011). Because there are other steroids in urine detected by the ELISA, the molarity is only an estimate meant to give some context to the amount of substituted 11-O-ETIO in the urine. Though we now are able to measure the amount of immunoreactivity of individual substituted forms of 11-O-ETIO separately, at the time these experiments were performed, immunoreactivy of separate forms of 11-O-ETIO were all measured together. Appendix D shows the amount of substituted 11-O-ETIO in the urine samples as measured by ELISA. The amounts in the EOG-tested solutions ranged from a minimum amount of 2.26×10^{-12} M 11-O-ETIO to a maximum of 8.89×10^{-10} M 11-O-ETIO.

5.2.4 Electro-olfactogram (EOG) recording

The EOG recording protocol was adapted from those previously published (Murphy et al., 2001; Bélanger et al., 2004) and has been described in detail in Chapter 4 (section 4.2.2). Briefly, non-reproductive females were anesthetized in 100 mg/l MS-222 (Finquel, Argent

Chemical, Redmond, WA, USA) and immobilized with gallamine triethiodide (150 mg/kg, Flaxedil, Sigma-Aldrich, Oakville, ON, Canada). Females were secured in a recording trough with dechlorinated water containing MS-222 flowing over the gills. The EOG was recorded differentially using glass capillary electrodes (tip diameter 80 – 100 μm) filled with 8% gelatin dissolved in 0.9% saline. Electrodes were bridged to Ag/Ag-Cl pellets via 3 M KCl. Signals were amplified, digitized (Powerlab, AD Instruments, Colorado Springs, CO, USA) and displayed on a computer running Windows Vista. EOG responses to odours were recorded as raw millivolt (mV) responses (for steroids) or normalized (for urine, to reduce some variability between individual test fish) by dividing by the average response to the standard 10^{-5} M L-alanine. Only fish that responded to the L-alanine standard were used.

To verify that these were not reproductive phase females, following recording of male urine responses, female gobies were sacrificed to calculate the gonadosomatic index (GSI), a measure of gonad weight as a percentage of total body weight, that is often used to estimate the reproductive status of fishes. A female round goby is considered reproductive if the GSI value of 8% or greater. It was determined that EOG responses were recorded only from nonreproductive females.

5.2.5 Data handling and analysis

The EOG responses to RM versus NRM urine samples were analyzed using Paired Student's t-tests (for all RM versus NRM data; Paired tests were used because each female was given an RM and NRM urine and those responses were compared) or regression analyses using GraphPad Prism 5. Data were normally distributed and displayed equal variance. For the synthetic steroids, each concentration was presented to a female at least 3 times and the responses were averaged for each concentration. Regarding the urine study, in a few instances,

female experimental fish were presented urine samples from the same donor males (see Appendix D). If a particular urine preparation was tested on two females (e.g. urine #38), these two responses were averaged to generate one average response to that particular urine. For example, responses to urine 38 by female -05 and -06 were averaged, and this value (average response to urine 38) was tabulated for each urine dilution that was tested (Appendix D).

5.3 Results

Only fish that responded to the positive control odour, L-alanine were used in this study. Female round gobies showed limited olfactory responses to the synthetic compounds tested in this study. EOG responses were not observed when either 10^{-12} M - 10^{-8} M 11-O-ETIO-17-s (5.1E, Fig 5.2) or 10^{-12} M - 10^{-8} M 11-O-ETIO-3-g (5.1C, Fig 5.3) were tested. Responses to 10^{-12} M - 10^{-8} M 3α,17β-dihydroxy-5β-androstan-11-one (5.1D) were variable. Of five tested fish, only one (F08-11-09) detected the compound consistently and in a dose-response manner (coded F08-11-09; Fig 5.4A). The female F08-11-09 was also the only fish to detect 3α,17β-dihydroxy-5β-androstan-11-one at more than one concentration (Fig 5.4A). This inconsistency in responses led to the large amount of variability shown in the averaged EOG dose-response curve (Fig 5.4B). One fish was tested for an EOG response to cortisone and cortisol, as male round gobies may be releasing these stress hormones during the period of isolation when urine collects in the bladder or when we are collecting waters for further testing (e.g. generation of methanol extracts as described in Chapter 4; Katare et al., 2011). The response of this fish to cortisol was low compared to other steroids (i.e. 0.4 mV EOG response to 10^{-8} M cortisol compared to 2.25 mV response to 10^{-8} M ETIO; Chapter 3; Murphy et al., 2001), and there were no responses to 10^{-8} M or 10^{-9} M cortisone (Fig 5.5). Generally, if an odour elicits a change in the EOG recording greater than 0.2 mV it is considered a response (Murphy et al., 2001).

80
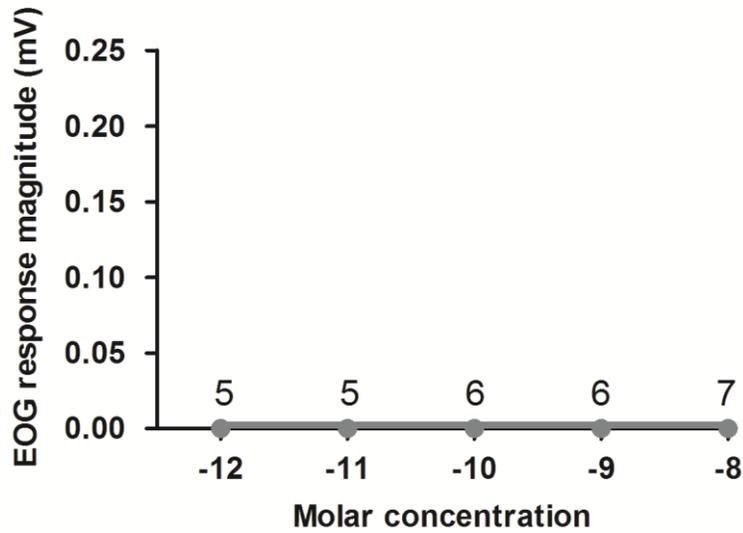
Fig 5.2: EOG dose-response relationship for the released steroid 11-O-ETIO-17-s (1E), recorded from non-reproductive female round gobies. There was no response to this steroid at any concentration tested. N for each concentration is listed.
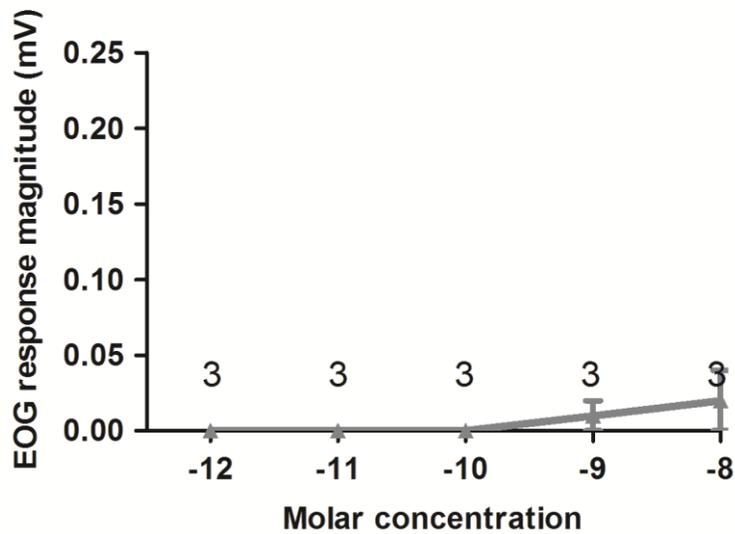
Fig 5.3: EOG dose-response graph for the released steroid 11-O-ETIO-3-g (Fig. 1C). Non reproductive female round gobies did not detect 11-O-ETIO-3-g, even at the highest concentration tested. N=3 for each concentration.
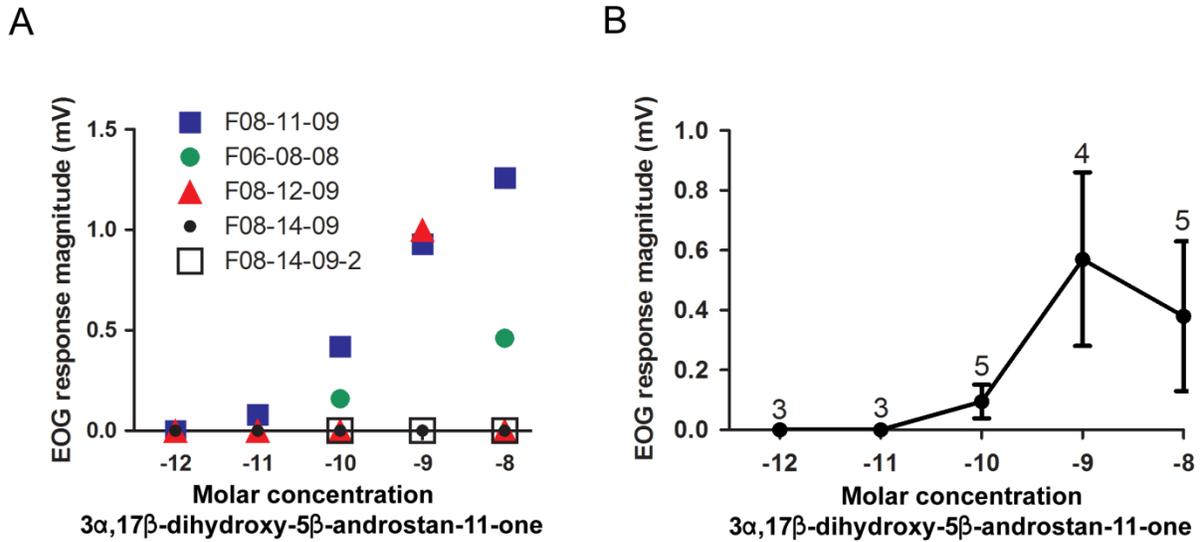
Fig 5.4: EOG responses recorded from female round gobies upon exposure to the intermediate 3α,17β-dihydroxy-5β-androstan-11-one (1D). (A) Graph shows EOG responses from the five females. Female F08-11-09 was the only fish to detect compound 1D at more than one concentration, and responded to it in a dose-response manner. Females F06-08-098 and F08-12-09 detected either 10^{-8} M or 10^{-9} M 3α,17β-dihydroxy-5β-androstan-11-one . The females F08-14-09 and F08-14-09-2 did not detect 1D at any concentration. (B) The averaged responses to 3α,17β-dihydroxy-5β-androstan-11-one (1D) from all 5 female round gobies shown in Fig 4A.
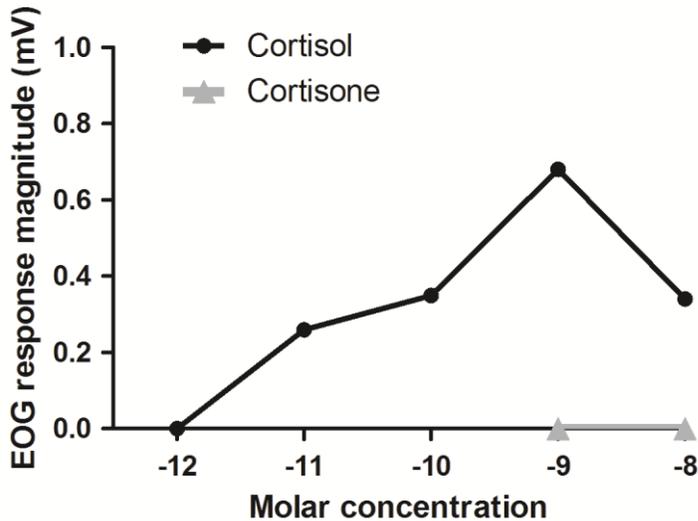
Fig 5.5: EOG responses taken from one female in response to cortisol and cortisone. The test fish was able to detect cortisol, but not cortisone.

Female round gobies exhibited robust EOG responses to RM and NRM urine samples, but overall there was no difference in female response to RM and NRM urines when the average responses to urine at each concentration were considered together (Fig 5.6; t=0.30, df=3, P=0.78). The detection threshold was approximately 1,000,000x diluted urine, which corresponds to a maximal concentration of 8.89 x 10^{-14} M substituted 11-O-ETIO. When testing the dose-response relationship, female responses to urine were highly variable and there was no difference in EOG magnitude between RM and NRM urine samples at any concentration (Fig 5.7). However, upon further analysis, it was found that the GSI values of the females that were responding to the urine were positively correlated with EOG response to NRM urine (Fig 5.8; r^2=94, P=0.0011). Surprisingly, the same relationship was not seen for RM urine (Fig 5.8; r^2=0.52, P=0.10).
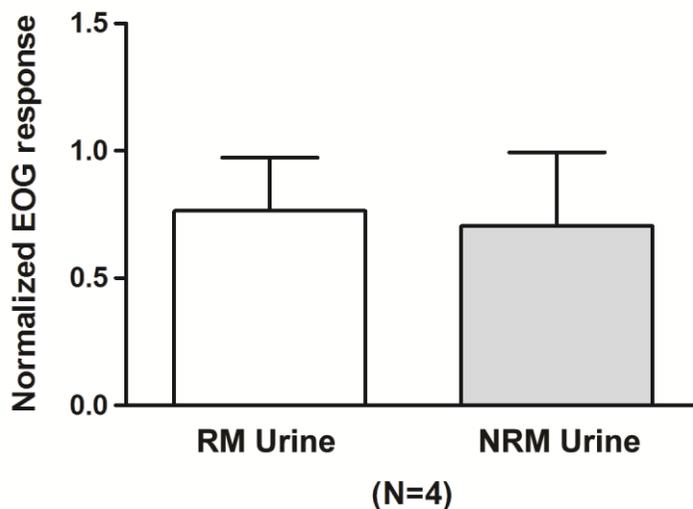
Fig 5.6: EOG responses from female round gobies to reproductive male (RM) and non-reproductive male (NRM) urines. There is no significant difference in response to RM and NRM urine when responses to all concentrations are considered together.
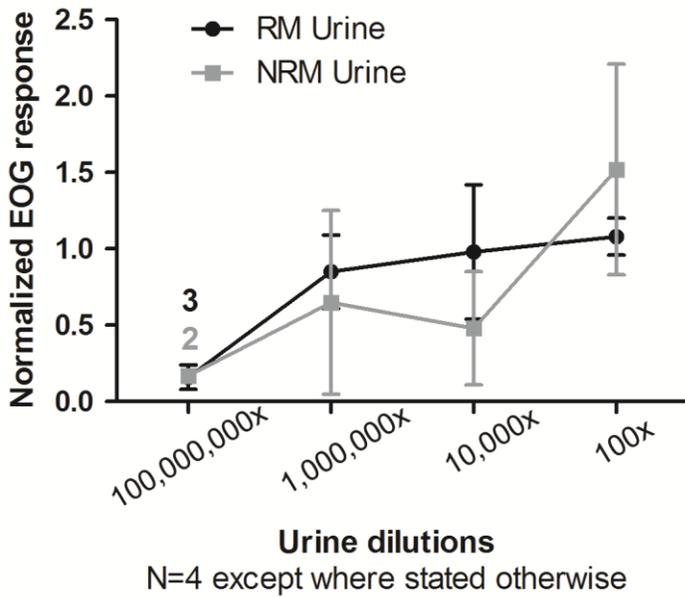
Fig 5.7: Normalized EOG responses to reproductive male (RM) and non-reproductive male (NRM) urine. There are no statistically significant differences in female response to RM on NRM urine.
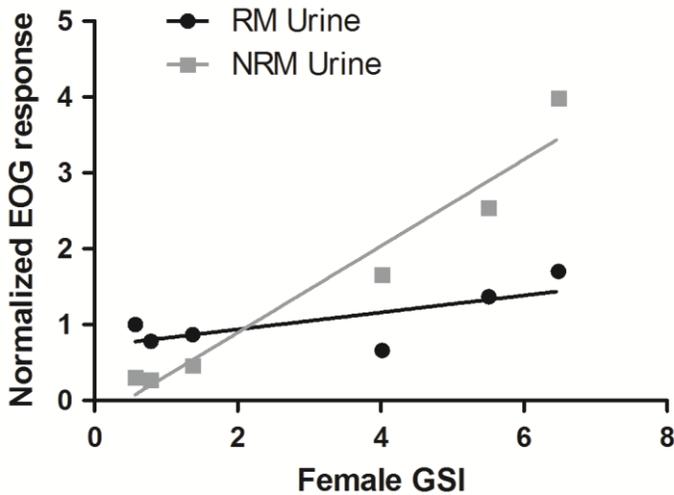
Fig 5.8: Relationship between female gonadosomatic index (GSI) and EOG response to urine samples. There is a significant positive correlation between female GSI and EOG response to NRM urine, but not RM urine. Urine samples were diluted 100x. All urine samples were used to produce this graph.

Table 5.1: The EOG responses to, and site of release of steroids released by male round gobies. Proposed routes of release are based on previous literature examining routes of release of odours. The substituted steroids are listed in decreasing order of abundance in which they are detected by ELISA in the urine (Katare et al., 2011). 11-O-ETIO-3-g has been tested before, but the sources of the steroid were different in the study be Bélanger (2003) than that used in this chapter.

Steroid	Proposed route of release	Detected by EOG?	Reference
11-O-ETIO	Gills	Yes	Chapter 4
11-O-ETIO-17-s	Urine	No	This study
11-O-ETIO-3-g	Urine	Yes No	Bélanger, 2003 This study
11-O-ETIO-17-g	Urine	Unknown*	
11-O-ETIO-3-s	Urine	Yes	Chapter 4

* Synthetic 11-O-ETIO-17-g analogue is not commercially available

5.4 Discussion

Male round gobies release a number of steroids that have a 5β-reduced and 3α-hydroxyl configuration (5β,3α) into the water where these may be detected by – and possibly be attractive to – females. Table 5.1 provides a summary of steroids tested. As described in Chapter 4, free 11-O-ETIO and 11-O-ETIO-3-s are stimulatory to female round gobies. The steroidal compounds tested in this study are not potent odourants to the round goby, and thus, are unlikely to be attractive releaser pheromones. The 11-O-ETIO-17-s (5.1E) is not detected, but it is the main contributor to the 11-O-ETIO immunoreactivity of RM urine, with 11-O-ETIO-3-g occurring as the second most abundant of the measured steroids (Katare et al., 2011). At this point the biological significance of this steroid (if any) is unknown. It is possible that steroids and their metabolites may function as pheromones even if they do not evoke EOG activity. Priming pheromones do not elicit behavioural responses, but instead lead to endocrine responses in the receiver (e.g. Sorensen et al., 2004), such as the resulting release of gonadotropin, and stimulation of sperm production in male goldfish upon exposure to free and sulfated 17α,20β-dihydroxy-4-pregnen-3-one (17α,20β-P and 17α,20β-P-20S). In the case of goldfish, they do

detect 17α,20β-P and 17α,20β-P-20S via EOG (Sorensen et al., 1995), but this does not necessarily mean that compounds with priming actions must evoke EOG responses. Sex steroids have the ability to be taken up by the gill epithelium, via sex hormone-binding globulin (Miguel-Queralt and Hammond, 2008), which presents an alternate route for steroids released into the water to affect a receiver if not through the olfactory system. However, there is not yet any direct evidence that an undetectable steroid could have priming effects.

The round goby also doe not detect 11-O-ETIO-3-g (5.1C); however, analysis by mass spectrometry (Appendix C) shows two peaks when this compound is run, indicating some impurity or breakdown of this test compound. If the steroid was impure or somehow damaged, that could account for the lack of response, as the round goby was previously shown to detect 11-O-ETIO-3-s to a sample that was custom synthesized for Dr. A. P. Scott by an earlier supplier (Bélanger, 2003). Different sources for this steroid could account for the differences in response between the earlier study and this one.

Responses to 3α,17β-dihydroxy-5β-androstan-11-one (5.1D) yield the most inconsistent results. This compound was tested because it may be an intermediary in the synthesis of substituted forms of 11-O-ETIO (see Fig 1, Katare et al., 2011). The lack of olfactory activity could be due to the fact that it was synthesized in our own lab, and there might have been some contamination. In addition, this compound is considered a highly reactive intermediate in the proposed biosynthetic pathway of some substituted forms of 11-O-ETIO and it is unlikely to be released (Katare et al., 2011). Therefore, I do not consider this compound to be a putative pheromone.

Responses to cortisol were only tested on one fish, which appeared to detect it, though at much lower magnitude than that of other steroids (Chapter 4; Murphy et al., 2001). Cortisone is

not detected at all by the test fish. Though the RM gobies are releasing some cortisol due to the stressful conditions of water collection (one male fish in 1 L of water), there is no significant increase in cortisol following injection of the males with salmon gonadotropin releasing hormone analogue (Katare et al., 2011), indicating that the olfactory potency of extracts of conditioned water from sGnRHa injected RMs (e.g. Chapter 3) is not due to the presence of cortisol.

Female round gobies exhibit robust EOG responses to urine collected from RM and NRM round gobies. There were no significant differences between female EOG responses to RM and NRM urine. The estimated molarity of immunoreactive substituted 11-O-ETIO contained in the urines is below the EOG detection threshold of free 11-O-ETIO and 11-O-ETIO-3-s (10^{-9} M, Chapter 3), even in the most concentrated urine samples tested, thus the urines are far more potent than individual synthetic steroids. The urine was collected from males that were not injected with sGnRHa to stimulate steroid production, and the stress of having their papillae tied may have caused them to reduce steroid production. Stress such as this has been shown to reduce steroid production (Garcia-Lopez et al., 2007). When the data were analyzed more closely, it became clear that the GSI of the female has some relationship to the olfactory response to urine, and in fact, is correlated positively with response to NRM, but not RM, urine. A confounding factor is the amount of substituted 11-O-ETIO in the urine samples, but no relationship between these amounts and EOG response is seen (data not shown). At this point, we have only measured 11-O-ETIO in the urine, and do not know what else may be in the urine that could account for this difference in female response, but urine contains a number of components such as ions (potassium, sodium, chloride), glucose and proteins (Erickson and Gingerich, 1986) that may affect the EOG response. The increase in GSI could indicate that the females are nearing reproductive status, and could have been experiencing increasing egg

production or vittelogenesis, though we do not know how that could affect their olfactory responses to urine. Pre-ovulatory reproductive female (i.e. eggs are ready for release but have not yet been released for fertilization) Mozambique tilapia (*Oreochomis mossambicus*) show greater EOG responses to urine from dominant males compared to subordinate males (Barata et al., 2008). Our results seem to be contrary to this, although if reproductive status proves to be a critical factor in female response, a better measure of reproductive status than GSI must be established. It would be beneficial to correlate GSI with the maturity of the ova and steroid levels in the female to create a clearer picture of reproductive status.

The average amount of immunoreactive substituted 11-O-ETIO in the urine samples is greater in the RM than the NRM urine (Appendix D). I have not seen a direct link between 11-O-ETIO immunoreactivity and EOG response from either urine or methanol extracted steroids. Increasing male release of 11-O-ETIO increases female response to methanol extracted male water (Chapter 3), but female EOG response to methanol extracts do not correlate with the amount of 11-O-ETIO contained in the extracts (data not shown). It is unknown exactly what in the NRM urine is causing the increased response from NRFs of a higher GSI. The RM urine does have higher levels of substituted 11-O-ETIO than NRM urine; however, the immunoreactive conjugate tested here (11-O-ETIO-17-s) is not detected by the OSNs in the round goby. The gobies do detect 11-O-ETIO-3-s, but this conjugate is not immunoreactive to the antibody used in this ELISA. When the antibody was generated, bovine serum albumin was substituted to 11-O-ETIO at the 3 position, leading to the generation of an antibody that does not recognize 11-O-ETIO that contains substitutions at the 3 position, but it will recognize substitutions where the sulfate or glucuronide groups are located at the 17 position (Katare et al., 2011); therefore it appears that the steroids that we are measuring using ELISA (prior to

sulfatolysis or glucuronidase treatment) are not necessarily the steroids that the round goby is capable of smelling. Analysis of the urine by Katare et al., (2011) showing that there is more of the 11-O-ETIO-17-sulfate than the 11-O-ETIO-3-sulfate in the urine, used urine from RM gobies that had been injected with sGnRHa. It is unknown if treatment with sGnRHa alters the composition of the male urine compared to what the composition would be normally. That is to say, under natural conditions, the 11-O-ETIO-17-sulfate may not be the predominant form of 11-O-ETIO released via the urine, and the quantities and timing of release may be important factors in any possible pheromonal activity of male urine or conditioned water.

There is one released conjugate of 11-O-ETIO that we have been unable to purchase, and that is 11-O-ETIO-17-g (Fig. 5.1F). This steroid is immunoreactive (i.e. it is detected by the ELISA) and is present in urine (Katare et al., 2011). Because we were unable to procure a synthetic form of 11-O-ETIO-17-g, I do not know if the female olfactory system can detect it.

The NRFs of a higher GSI (> 4%, approaching "reproductive", which is 8% or greater, Bélanger et al., 2004) have a greater EOG response to NRM urine than RM urine, while the opposite was true of the very low (< 4%) GSI females. Why these higher-GSI females show a greater response to NRMs is unclear. Urine samples used were classified as coming from RMs or NRMs based on the GSI values of the males which were calculated after collecting the urine and sacrificing the males (Yavno and Corkum, 2010). It is possible that NRMs classified as such may have just released sperm or guarded eggs and the urine composition may have been altered in a way to increase the olfactory potency. It could be that the females are attracted to males that have fertilized eggs because the eggs are a possible food source or because it is indicative of the male's reproductive success (e.g. a male that has already fertilized eggs has already proven his fitness).

It is possible that the urine alone isn't necessarily a pertinent cue to the female during mate selection, when the animals are interacting from a short distance. For example, Yavno and Corkum (2010) found that type of urine (RM or NRM) had no effect on the amount of time spent by reproductive females near an experimental nest containing a model of a male fish though it must be noted that in this study the females were in fairly close proximity to the males, and olfactory cues may not be as important under these conditions. In addition, the RMs in the Yavno and Corkum (2010) study might not have been releasing elevated steroids, possibly because they were not treated with sGnRHa and they were under captivity stress. Urine alone may not be the source of reproductive pheromone(s), but that other compounds, perhaps released via the gills or in the feces, might also be important for mate selection. Clearly, the relationship between male signal and female response is complex and requires more study.

The final possibility is that the RMs caught in the field and sampled for urine just weren't producing pheromonal compounds that would elicit high olfactory responses. The stress of captivity has been shown to negatively impact steroid production and gonadal maturity (Garcia-Lopez et al., 2007). This is why in previous studies (Chapter 3; Katare et al., 2011) the hypothalamic-pituitary-gonadal axis was stimulated with sGnRHa.

It has been observed that some RM round gobies may be producing greater quantities of attractive pheromonal compounds while others are producing very little, leading to the classification of some males as "studs" and others as "duds" by Qureshi (2008). The biological "dud" still exhibits the secondary sexual characteristics identifying it as reproductive, and it has a high GSI value, but for some undetermined reason, it is not producing pheromones. When males were injected with sGnRHa and the methanol extracts were collected, overall sGnRHa increased 11-O-ETIO release by a factor of 2.29 (as measured by ELISA), but 2 out of 5 treated RMs did

not increase their release of 11-O-ETIO (Quereshi, 2008). Angling may select for the duds, as nest guarding round gobies (the males assumed to release the most pheromone) do not feed during this time (Miller, 1984; Charlebois et al., 2001); therefore the RMs that are being caught are feeding and may have already left their nests for some reason, for example, if they have already fertilized and guarded eggs (Quereshi, 2008).

To conclude, none of the synthetic analogues of steroids released by RM round gobies tested here present themselves as good candidate releaser pheromones, compared to those released compounds tested in Chapter 4 (11-O-ETIO and 11-O-ETIO-3-s). There certainly could be additional compounds released by males that we have yet to identify, and we haven't been able to test the olfactory potency of all the identified compounds (e.g. 11-O-ETIO-17-g, which is not commercially available). In addition, urine is a composite of many compounds, and may not be the best test to use in behavioural or olfactory testing, as the response by females to these stimuli is difficult to interpret.

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Chapter 6:

The effects of prostaglandin $F_{2\alpha}$ on female round goby olfactory responses

6.1 Introduction

Prostaglandins (PGs) are a group of fatty-acid derived lipid compounds that have a variety of biological functions. In fishes, PGs are best known for their ability to initiate female spawning behaviours (for a recent review, see Munakata and Kobayashi, 2010). Increased circulating levels of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at the time of ovulation stimulate female spawning behaviours in goldfish, (*Carassius auratus)*, (Stacey, 1976; Stacey and Peter, 1979; Stacey and Goetz, 1982), and $PGF_{2\alpha}$ injection stimulates spawning behaviour in two fishes of the order Perciformes: a cichlid (*Cichlasoma bimaculatum*, Cole and Stacey, 1984), and the paradise fish (*Macropodus opercularis*, Villars et al., 1985). In the goldfish, $PGF_{2\alpha}$ is also a component of the pheromone released by females immediately preceding ovulation, and stimulates reproductive behaviours in males (Sorensen et al., 1988).

In addition to these roles in fish reproduction (e.g. Sorensen and Goetz, 1993), PGs have a variety of other functions in other organisms. For example, during nociception and pain in mammals, PGs mediate inflammatory responses by sensitizing neurons (reviewed by Meves, 2006; Premkumar et al., 2006). This sensitizing effect occurs through binding with G-protein coupled receptors (Narumiya, 2009), and PGs act directly on ion channels, lowering their resting voltage and thus increasing the probability of depolarization (Meves, 2006). In embryonic rat dorsal root ganglion cells, PGs affected the production of cAMP (e.g. Nicol and Cui, 1994; Cui and Nicol, 1995). G-protein coupled receptors, ion channels and cAMP are possible sites for the sensitizing actions of PGs during the olfactory transduction, as well. The olfactory receptor is a G-protein coupled receptor (Buck and Axel, 1991) and olfactory signal transduction relies upon the activation of a cyclic-nucleotide gated ion channel (Firestein, 2005). For example, PGs may

affect the generation of action potentials or the number of responding cells in the olfactory epithelium,

Hormonal modulation of olfactory sensory neurons in the periphery is not unheard of. Some hormones affect responses of olfactory sensory neurons (OSNs). Recordings taken from the olfactory epithelium (electro-olfactogram, EOG) of axolotls (*Ambystoma mexicanum*) were inhibited in the presence of gonadotropin releasing hormone (Park and Eisthen, 2003). In addition, the satiation hormones leptin and insulin reduced EOG responses recorded from rats in response to the food odour isoamyl acetate (Savigner et al., 2009).

In brown bullhead catfish (*Ameiurus nebulosus*) the amplitude of the EOG is correlated with the number of responding olfactory sensory neurons (Koce and Valentinčič, 2000), therefore we could postulate an increased EOG response magnitude if PGs are capable of sensitizing neurons within the olfactory epithelium. For example, PG sensitization could lead to the recruitment of olfactory sensory neurons that would respond to an odour.

The round goby (*Neogobius melanostomus*) was used to perform a pilot study investigating the possibility that $PGF_{2\alpha}$ could act physiologically to increase the olfactory response of female fishes. Female round gobies have been seen to exhibit a greater EOG response to male odours when they are reproductive versus non-reproductive (Bélanger et al., 2004). We hypothesized that this increased olfactory sensitivity might be related to higher levels of $PGF_{2\alpha}$ associated with reproductive maturity (Stacey and Goetz, 1982).

6.2 Methods and Materials

6.2.1 Collection and housing of fish

Non-reproductive female round gobies were angled from Lake Erie in Leamington, Ontario and transported to the University of Windsor where they were housed in the animal care

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facilities in June through August, 2009. Fish were held in flow through troughs supplied with

dechlorinated municipal tap water, held at ambient temperatures on a 16:8 hr photoperiod. Fish

were fed once daily with commercial flake fish food (Aquatic Eco-systems, Apopka, FL, USA).

Females were housed separately from male round gobies.

6.2.2 Odour preparation

All compounds that were tested as odours were purchased from Sigma-Aldrich (Oakville,

ON, Canada) or Steraloids (Newport, RI, USA). For this pilot study, an odour blend containing

representatives of different odour classes was used. It contained: 10^{-5} M L-alanine (an amino

acid), 10^{-6} M taurocholic acid (TCA, a bile acid), and 10^{-8} M 11-oxo-etiocholanolone (11-O-

ETIO, an androgen and putative pheromone). L-alanine and TCA were prepared initially as 10^{-2}

M stock solutions in dechlorinated water, then diluted serially in dechlorinated water to their

final test concentrations. These were prepared fresh daily. 11-O-ETIO was prepared as a 10^{-2} M

stock solution in 100% methanol and stored at -20°C until use. On test days, 11-O-ETIO was

diluted serially from the stock solution to its final test concentration (10^{-8} M). The odours were

combined to make a blend so that the final concentration of each individual odour in the blend

was maintained as mentioned above.

6.2.3 Recording of field potentials from the olfactory epithelium (electro-olfactogram, EOG)

The method for recording summed generator potentials from the surface of the olfactory

epithelium of round gobies (electro-olfactogram, EOG) has been described elsewhere in detail

(Murphy et al., 2001; Bélanger et al., 2004; Chapter 4 of this thesis). The EOG was recorded

from anesthetized, immobilized non-reproductive female round gobies by placing a glass

capillary electrode (filled with 8% gelatin in 0.9% saline) in the anterior naris. The reference

electrode was placed lightly on the skin surface near the anterior naris. A glass odour delivery

tube was placed over the posterior naris and provided the olfactory epithelium with either a continuous flow of dechlorinated water or odours (introduced into the background flow of dechlorinated water periodically as 5 second pulses). At least 2 minutes were provided between odour exposures in order to avoid adaptation of the olfactory sensory neurons.

6.2.4 Treatment with prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and test protocol

Two experimental treatments and two types of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) were used in this study. In Experiment One, the $PGF_{2\alpha}$ was dripped directly on the olfactory epithelium in the exposure, as previously performed for GnRH modulation by Park and Eisthen (2003), and in Experiment Two, female round gobies were treated by $PGF_{2\alpha}$ injection during EOG recording.

Experiment One: The sensitization of olfactory sensory neurons by $PGF_{2\alpha}$ perfusion directly over the olfactory epithelium was examined. The $PGF_{2\alpha}$ for perfusing over the naris was purchased from Sigma-Aldrich (#P0424), dissolved at 5 µg/10 µl in 0.9% saline, aliquoted and stored at -20°C. For testing, individual $PGF_{2\alpha}$ aliquots were diluted serially in dechlorinated water to 10^{-8} M $PGF_{2\alpha}$, since the $PGF_{2\alpha}$ does not elicit an EOG response at this concentration in the round goby (Murphy et al., 2001).. The 10^{-8} M $PGF_{2\alpha}$ was delivered to the olfactory epithelium continuously and the odour blend was delivered in 5 second pulses at 2, 4 and 6 minutes of $PGF_{2\alpha}$ exposure. The $PGF_{2\alpha}$ did not evoke EOG responses (Murphy et al., 2001; A. Laframboise, personal observation). Two animals were used in this pilot experiment.

Experiment Two: The ability of $PGF_{2\alpha}$ to increase female olfactory response after systemic injection was investigated by using a commercial product, Lutalyse® (Vet Purchasing Company Ltd, www.vpcl.on.ca), which is $PGF_{2\alpha}$ in a stabilizing vehicle, and is injected without additional preparation. We switched to Lutalyse due to its reliability and stability for Experiment Two on the recommendation of Dr. Norm Stacey (U. of Alberta). The gobies were secured on the EOG

rig, and injected into the dorsal musculature with 10 µl of Lutalyse (containing approximately 0.05 mg of $PGF_{2\alpha}$) solution. The odour blend was tested once every 10 minutes for 30 minutes before injection with Lutalyse, and then once every10 minutes following the injection for up to 60 minutes post-injection. Experiment Two was performed on 5 fish. Both experiments were performed in July 2009.

6.2.5 Data handling and analysis

The EOG responses were recorded as millivolts (mV). For Experiment Two, the responses by 5 females injected with Lutalyse were averaged for data analysis. Data were analyzed using either linear regression analysis or repeated measures ANOVA (where appropriate) with GrapPad Prim 5.

6.3 Results

The purpose of this pilot study was to investigate whether treatment with $PGF_{2\alpha}$ has an effect on female round goby olfactory responses. In Experiment One, the olfactory epithelium of the fish was perfused with $PGF_{2\alpha}$ in an attempt to sensitize the olfactory sensory neurons directly. This pilot experiment was performed on two fish. Fish 1 showed an immediate rise in EOG response 2 minutes after starting $PGF_{2\alpha}$ perfusion of the nasal cavity, after the 2 minutes, the response remained stable at this elevated level for EOG responses recorded during minutes 4 and 6 of the $PGF_{2\alpha}$ treatment (Fig 6.1). The response of Fish 2 was the opposite; the response dropped at minute 2 of the $PGF_{2\alpha}$ treatment, dropped again during minute 4, and then remained level during minute 6 (Fig 6.1).
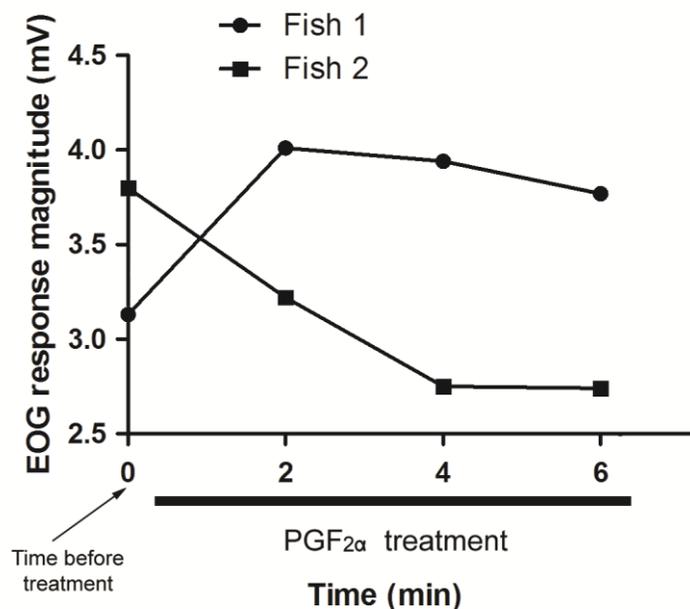
Fig 6.1: Experiment One. EOG responses from two female round gobies in response to an odour blend (10^{-5} M L-alanine, 10^{-6} M TCA, 10^{-8} M 11-O-ETIO) before and during treatment of the olfactory epithelium with 10^{-8} M $PGF_{2\alpha}$. The time = 0 response was recorded before beginning the $PGF_{2\alpha}$ perfusion. The first response was recorded 2 minutes after perfusion with $PGF_{2\alpha}$ began (time 2 on figure 6.1)

In experiment two, $PGF_{2\alpha}$ was injected intramuscularly into the female and the olfactory response to the odour blend was monitored for one hour. For this pilot study, five fish were injected with $PGF_{2\alpha}$ and one was injected with saline. Female round goby EOG responses showed an increasing trend for 50 minutes following injection with $PGF_{2\alpha}$ (Fig 6.2), however the slope of line connecting the response magnitude values was not significantly different from zero (r^2=0.38, P=0.30), nor was it significantly different from the slope of the line for the EOG responses that were recorded 30 minutes preceding treatment (F=0.029, df=2, P=0.097). The EOG responses recorded from single fish injected with saline (negative control) varied wildly over time. As a further analysis, time bins were created by combining: (a) all the data for the 30 minutes pre-injection (b) the data for 10 – 30 minutes post $PGF_{2\alpha}$ injection (c) the data from all

fish for the 40 – 60 minutes post $PGF_{2\alpha}$ injection (Fig 6.3). When given the odour blend pre-injection, females had an average EOG response of 2.88 mV, which was not significantly different from responses during the first 30 minutes post-injection, which were 2.92 mV on average. EOG responses increased by nearly 1 mV, to 3.77 mV, in the time from 40 – 60 minutes post-injection, but this was not significantly different from responses during the other two time periods (F=2.53, df=14, P=0.12). There was a trend for odour responses to increase over time with $PGF_{2\alpha}$ treatment.
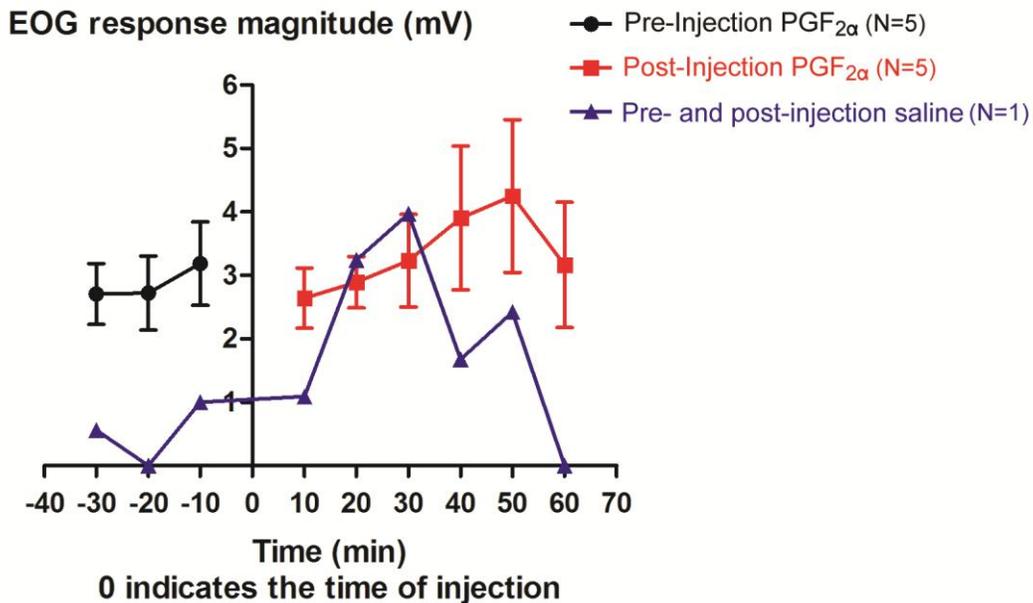
Fig 6.2: Experiment Two. EOG recordings in response to the odour blend (10^{-5} M L-alanine, 10^{-6} M TCA, 10^{-8} M 11-O-ETIO) taken from female round gobies before and after injection of $PGF_{2\alpha}$. Times -30 to -10 minutes indicate the three responses prior to injection, the injection occurred at time 0, and 10 – 60 minutes were recorded after the injection. There was a non-significant increase in response to the odour blend over time. The blue line indicates the responses recorded from one fish injected with saline.
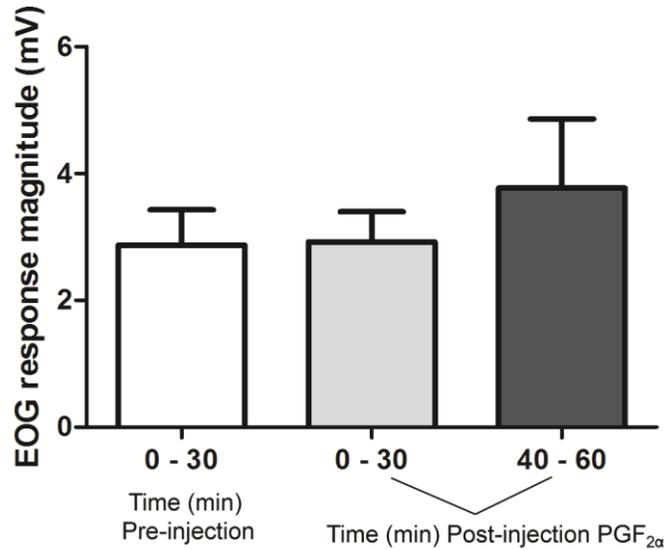
Fig 6.3: Experiment Two. Pooled EOG responses to odour blend (10^{-5} M L-alanine, 10^{-6} M TCA, 10^{-8} M 11-O-ETIO) recorded from round goby females 30 minutes before injection with $PGF_{2\alpha}$ (white bar), 0 – 30 minutes after injection (light grey bar), and 40 – 60 minutes post injection (dark grey bar). There was a non-significant increase in EOG responses to the odour blend between 40 and 60 minutes post treatment with $PGF_{2\alpha}$. N=5

6.4 Discussion

Prostaglandins have a variety of biological effects, during reproduction and otherwise. $PGF_{2\alpha}$ induces reproductive behaviours in both female and male goldfish (Stacey et al., 2003) as well as females of some other fish species (Cole and Stacey, 1984; Villers et al., 1985; reviewed by Munakata and Kobayashi, 2010). Nothing is known, however, about the effect of $PGF_{2\alpha}$ in the round goby. Treating female round gobies with $PGF_{2\alpha}$ does not significantly increase their olfactory response, though the results suggest that olfactory generator potentials may be affected. With refinement of the technique and additional replicates, one – or both – techniques may yield better results. In experiment one, $PGF_{2\alpha}$ was used in an attempt to sensitize olfactory sensory neurons from the luminal surface of the olfactory epithelium. It is difficult to speculate as to

why the results for the two fish were contrary and it may be helpful to test this procedure again on additional fish.

Research using embryonic rat dorsal root ganglion (DRG) cells grown in culture shows that treating the cells with prostaglandin E_2 (PGE_2) increases the number of bradykinin-induced action potentials (Nicols and Cui, 1994). It could be that $PGF_{2\alpha}$ does not exert the same sensitizing effect on neurons as PGE_2, but this has never been investigated. Structurally, PGE_2 and $PGF_{2\alpha}$ are very similar and they are both used in mammals to induce labor (Kelly et al., 2009). PGE_2 has a range of effects on different cell types (reviewed by Meves, 2006), but PGE_2 was not used here because it is $PGF_{2\alpha}$ that has the established effects on fish reproduction (Stacey et al., 2003; Munakata and Kobayashi, 2010). Perhaps the olfactory epithelial perfusion method of treatment should be repeated with PGE_2 to investigate whether this type of PG can exert a sensitizing effect on olfactory sensory neurons as with other types of neurons. Additionally, the studies performed on rat DRG sensory neurons measured responses of individual cells (Nicols and Cui, 1994; Cui and Nicols, 1995), while the EOG records field potential changes (from neuronal ensembles). It is possible that not all cells in the olfactory epithelium respond to PGs with sensitization; PGs have the ability to act on G-protein coupled receptors or directly on ion channels (Meves, 2006). Since PGs do not elicit EOG responses directly (it is not an odorant; Murphy et al., 2001), we can assume that OSNs do not contain G-protein coupled receptors for PGs, making ion channels a more likely site of action for PGs in the olfactory epithelium, but even if PGs bind onto ion channels, you would expect to see a change in the EOG. The sensitizing effect, if it exists, could be diluted by the fact that the EOG records responses from so many cells, and the PG may be affecting the activity only of pheromone receptors, and we tested an odour blend. One OSN could be affected, while another

is not, and since the EOG records responses from thousands of cells, the net result would be no change. This type of test would best be performed when recording from single, or at least, fewer cells and using fewer odours. In fact, the modulatory effects of leptin and insulin (Savigner et al., 2009) and PGE2 (Nicols and Cui, 1994; Cui and Nicols, 1995) on OSNs were discovered by recording from single neurons, not ensembles as in the EOG recording.

$PGF_{2\alpha}$ is known to induce spawning behaviours in female fishes within 1 hr of injection, though in goldfish it can happen as quickly as 30 minutes (Stacey, 1976; Cole and Stacey, 1984; Villers et al., 1985). Unfortunately, there is no evidence that injecting a female fish with $PGF_{2\alpha}$ can increase the fish's olfactory epithelial response to male pheromones. It is likely that the behavioural effects of $PGF_{2\alpha}$ in goldfish are due to its actions in the brain; in fact, injecting $PGF_{2\alpha}$ directly into the brain is more effective at inducing spawning behaviours than intramuscular or intraperitoneal injection, though these other routes of administration do work (Stacey and Peter, 1978). Central effects of $PGF_{2\alpha}$ have been seen in male goldfish as well. Waterborne exposure to $PGF_{2\alpha}$ has a neurogenic effect in the male goldfish diencephalon, and it increases brain levels of gonadotropin releasing hormone and choline acetyltransferase, which are associated with the neuroendocrine and motor-behavioural changes seen during the reproductive phase (Chung-Davidson et al., 2008). It is also possible that no one has had the opportunity of investigating the possibility of $PGF_{2\alpha}$ having a sensitising effect on neurons in the periphery, particularly since most of the research on prostaglandins has focused on goldfish, and there has been virtually no interest in female goldfish responses to male odours (Stacey and Sorensen, 2002); this is simply given the nature of the goldfish mating system, as females release pheromones which are attractive and stimulatory to the males. There is evidence that treatment via androgen implantation can increase the EOG response of male tinfoil barbs (*Barbonymus*

schwanenfeldii, Cardwell et al., 1995) and male redtail sharkminnow (*Epalzeorhynchos bicolour*, Belanger et al., 2010) to pheromones, but similar evidence in female fishes is lacking. Should this experiment be repeated successfully, ours could be the first study to show an increased peripheral olfactory response in female fish following treatment with $PGF_{2\alpha}$.

It would also be interesting to attempt recording EOGs following injection with $PGF_{2\alpha}$ in the goldfish or a similar cyprinid, simply because we know that $PGF_{2\alpha}$ works to increase spawning behaviours in these fishes. We still do not know if $PGF_{2\alpha}$ has this behavioural effect on the round goby, though initial trials indicate that injecting non-reproductive female round gobies with Lutalyse does increase the strength of their behavioural response to fractionated RM round goby conditioned-water (Kereliuk, personal communication). A logical first step in this experiment is to establish the behavioural effect of $PGF_{2\alpha}$ on the female round goby, and then to continue with the electrophysiology. This can be done quite easily by following the methodology outlined by Cole and Stacey (1984), where female round gobies are injected with Lutalyse, placed with reproductive males, and then monitored for spawning behaviours, which have been characterised (Meunier et al., 2009). The difficulty here is to ensure that the fish are fully acclimated to the lab and that the proper environmental conditions are met. Though this proposed experiment would be quick to run, the set-up for it would probably involve several weeks of preparation to ensure that the conditions are right and that the fish are not under any stress.

It appears that the possible effect of $PGF_{2\alpha}$ on olfactory responses does not occur until after 30 minutes and may take even longer. EOG recordings from the round goby can often be taken for several hours, but tend to degrade. It may be necessary to inject the fish with Lutalyse prior to beginning EOG recordings, but given the natural variability fish-to-fish, it would be very

difficult to see an effect without each fish having its own pre-injection period to serve as a control. It is probably best to continue with the methodology described here, but it may need to be replicated many times in order to get enough fish surviving long enough to complete the trial.

Rapid – within minutes or hours, not days or weeks – steroid effects on the olfactory system have never been demonstrated in any species of fish (previous studies have used long-term exposure of a week or more; Cardwell et al., 1995; Belanger et al., 2010), but rapid steroid effects of 11-ketotesosterone and estradiol and have been seen in the auditory system of gulf toadfish (*Opsanus beta*; Remage-Healey and Bass, 2006) and the plainfin midshipman fish (*Porichthys notatus*, Remage-Healey and Bass, 2004). The potential to demonstrate something similar in the olfactory system is highly exciting, and such a study would be incredibly novel.

In conclusion, though these pilot experiments failed to find a significant effect of $PGF_{2\alpha}$ on female olfactory responses, there is an opportunity for continued investigation in this area, as the results were promising. The experiments described here – perfusion or injection with $PGF_{2\alpha}$ – present themselves as an interesting direction of future research of a highly novel and exciting nature.

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Chapter 7:

Preliminary investigation of primer pheromone effects of reproductive male round gobies

on female round gobies

7.1 Introduction

It has long been known that fishes employ hormonal products released into the water as pheromones. Recently, Stacey and Sorensen (2006) defined pheromones as "*a substance, or mixture of substances, released by an individual, which evokes a specific and adaptive response in conspecifics, the expression of which does not require learning*". Moreover, reproductive pheromones can be further divided into two types based on their actions: releasers and primers. Primer pheromones do not evoke behavioural responses, but instead induces changes in the endocrine system, while releaser pheromones elicit rapid behavioural effects (Wilson and Bossert, 1963).

For example, directly preceding ovulation, the female goldfish (*Carassius auratus*) releases a primer pheromone (referred to as "pre-ovulatory") consisting of $17\alpha,20\beta$-dihydroxy-4-pregnen-3-one ($17\alpha,20\beta$-P), $17\alpha,20\beta$-P-20β-sulfate ($17\alpha,20\beta$-P-s) and androstenedione (AD). When male goldfish are exposed to this pre-ovlulatory primer mixture released by the females, the males respond by increasing levels of serum gonadotropin (which stimulates the gonad to synthsize steroids), as well as milt volumes (Kobayashi et al., 1986; Stacey et al., 1989; Sorensen et al., 1995; Zheng and Stacey, 1997). However, following ovulation, the female releases prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolite 15-keto-$PGF_{2\alpha}$ (15K-$PGF_{2\alpha}$) into the water, where these comprise the post-ovulatory releaser pheromone, which stimulates spawning behaviours in the male (Sorensen et al., 1988).

This pheromone system is very well-characterized in the goldfish, but pheromones in other fishes have not been well-investigated. Primer pheromones in particular have received little attention. There is evidence for primer pheromones in salmonids such as the rainbow trout, *Oncorhynchus mykiss* (Olsén and Liley, 1993), Kokanee salmon, *Oncorhynchus nerka* (Liley et

al., 1993), and Atalantic salmon, *Salmo salar* (Waring et al., 1996; Olsén et al., 2001). Additionally, primer pheromones have been investigated in one other species of cyprinid fish, *Barilius bendelisis* (Bhatt and Sajwan, 2001). Apart from these studies, the existence of primer pheromones has not been expressly investigated in other fish species, including any species of the order Perciformes. A few studies have looked for releaser pheromones (e.g. Sorensen et al., 2004), but there has been no published report of a compound with primer functions in a percid fish.

The round goby (*Neogobius melanostomus*, order Perciformes, family Gobiidae) is an invasive species in the Laurentian Great Lakes system, where it has had negative impacts on native species and ecology (Charlebois et al., 2001). Given that the round goby inhabits dark, turbid environments, it has been postulated that reproductive males (RMs), who establish nests and provide parental care, could employ reproductive pheromones to attract females into nests to deposit their eggs. Previously, we have found evidence for releaser pheromones at work in the round goby; for example, reproductive females (RF) have been found to be attracted to RM conditioned water (Bélanger et al., 2004; Gammon et al., 2005). RMs that have been injected with salmon gonadotropin releasing hormone analogue (sGnRHa) release into the water several novel 5β-reduced androgens - 11-oxo-etiocholanolone (11-O-ETIO), 11-O-ETIO-17-sulfate (11-O-ETIO-17-s), 11-O-ETIO-3-glucuronide (11-O-ETIO-3-g), 11-O-ETIO-3-sulfate (11-O-ETIO-3-s), and 11-O-ETIO-17-glucuronide (11-O-ETIO-17-g) (Katare et al., 2011): Female round gobies detect 11-O-ETIO and 11-O-ETIO-3-s, but not the other released steroids. The detected steroids are good candidates for releaser pheromones, but what of the undetected steroids? RMs release them (sometimes in great quantities), and though they are not detected by the olfactory system, they might still function as primer pheromones. The purpose of this experiment was to

create and test an experimental protocol for evaluating the possible primer effects of round goby male conditioned water (containing free and substituted 11-O-ETIO) on the female endocrine system. As an endpoint, we tested testosterone released into the water by female round gobies following exposure to the conditioned water of RM gobies. Testosterone has been shown to be increase prior to ovulation in at least one species of the order Perciformes, the flounder, *Pseudopleuronectes americanus* (Campbell et al., 1976). Because this study was designed to be a rapid, initial investigation, we chose to measure testosterone because our lab was capable of measuring it using an established method for an enzyme linked immunosorbant assay.

7.2 Methods and Materials

7.2.1 Fish collection and housing

Male and female round gobies were angled from the Detroit River at Windsor, Ontario, Canada in July, 2008, during their breeding season. Reproductive male (RM) round gobies exhibited prominent secondary sexual characteristics such as dark nuptial colouration and puffy cheeks (MacInnis and Corkum, 2000). Non-reproductive males (NRM) and females (NRF) are not sexually dimorphic, and were differentiated based on the shape of their urogenital papillae. Males have a longer, pointed papilla, while females have a shorter papilla with the appearance of two lobes (Miller, 1984). Males and females were housed separately at the University of Windsor animal care facilities, in flow through aquaria with dechlorinated municipal tap water at ambient temperature ($20 - 24^{o}$C) on a 16 h light:8 h dark photoperiod. Round gobies were fed daily with commercial flake food (Aquatic Eco-systems, Apopka, FL, USA). All experiments performed were in accordance with the Canadian Council on Animal Care guidelines.

7.2.2 Generation of male conditioned water

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Male conditioned water was prepared by combining two protocols previously described (Gammon et al., 2005; Katare et al., 2011). We created three stimuli: RM conditioned water, NRM conditioned water, and dechlorinated water which had no fish odour. For production of the RM conditioned water, two RM round gobies were removed from their tank, lightly anesthetized with 0.05% 2-phenoxyethanol (Sigma-Aldrich, Oakville, ON, Canada) and injected with 20 µg/kg of sGnRHa, Ova-RH, Syndel Laboratories, Qualicum Beach, BC, Canada) diluted in 0.9% saline, then placed individually in glass jars containing 1 l of dechlorinated water with an airstone and left for 4 hours (Gammon et al., 2005). To create the NRM conditioned water, two NRM gobies were treated the same way, except that these were injected with 0.9% saline rather than sGnRHa. After 4 hours the males were removed and the conditioned water was collected. All of the injections were performed by undergraduate assistant Miss Zena Alyasha'e. Odour stimuli presented to females were pooled from two males. 125 ml was collected from each jar; the two samples from RM gobies were mixed together to create 250 ml of RM conditioned water while the two samples from the NRMs were mixed together for 250 ml of NRM conditioned water.

7.2.3 Exposure of females to male conditioned water

In order to test the possibility that males discharge pheromones with primer effects on the female round gobies, we exposed NRFs to the conditioned water generated by RMs injected with sGnRHa and NRMs injected with saline and tested for changes in the levels of testosterone in the females. The conditioned water treatment protocol was based on that of Bhatt and Sajwan (2001), and Sorensen et al. (2005), and was developed with the help of Dr. Alexander Scott (CEFAS UK). For exposing the female to male conditioned water, NRF gobies were placed individually in plastic buckets containing 1 l of dechlorinated water and an airstone. Females

were left for 1 hour to acclimate. Following acclimation, females were removed and placed into fresh buckets containing 950 ml of dechlorinated water mixed with 50 ml of one of the conditioned water treatments (RM or NRM conditioned water). The amount of conditioned water used was above levels that have previously been shown to induce behavioural responses in round gobies (Gammon et al., 2005; Belanger et al., 2006). For example, Gammon et al., (2005) observed behavioural responses from females exposed to 500 ml of water added to their 20 l tank. Here we exposed females in only 1 l of water to 250 ml of stimulus. Once the females had been moved into the conditioned water treatment, the water that held them during acclimation (pre-exposure female holding water) was collected for steroid analysis. The females were immersed in the male conditioned water for 3 hours, then they were removed to buckets containing fresh dechlorinated water for a final 2 hours, and this water was collected for analysis of post-exposure steroid levels (post-exposure female holding water). Only the water that held the females for the final 2 hours was collected, so that steroids in the male conditioned water would not interfere with the measurement of steroids released by the females. The entire experiment - the collection of the male conditioned water, the immersion of females in this water, and collection of the female conditioned water – was conducted on the same day. The males were injected in the morning, the male conditioned water was collected in the afternoon and the acclimated females immediately immersed into this male conditioned water. Every day, at least one female round goby was exposed to each of the three experimental stimuli. The experiment was replicated five times so that in total, 10 males were injected with sGnRHa or saline to collect cues (two males injected each time and waters pooled) which were tested on a total of five females each.

7.2.4 Water preparation and steroid analysis

Female holding water was collected before and after exposure to male conditioned water to measure the release of testosterone (T). Water preparation followed the procedure of Katare, et al., (2010), and these ELISA analyses were performed by Dr. Yogesh Katare. Steroids were extracted from water by running them through activated Seppak C18 cartridges (Waters, Milford, MA, USA), which were washed with 5 ml of distilled water before the steroids were eluted with 5 ml of methanol. Testosterone in the methanol extracts of female holding water was measured using enzyme-linked immunosorbant assay (ELISA) using a similar procedure to Katare et al. (2010). A solution of testosterone-BSA conjugate was used to coat Pierce amine-binding maleic anhydride activated plates (Thermo Scientific Inc.), following by incubation with primary antibody (provided by Dr. Alexander Scott), then secondary antibody.

7.2.5 Data analysis

NRF round goby release rates of T before exposure to male conditioned water were compared to T release rates after exposure to the male conditioned water using paired Students t tests. Data were analysed using GraphPad Prism 5.

7.3 Results

NRF round gobies were exposed to one of two treatments: conditioned water from RMs injected with sGnRHa, conditioned water from NRMs injected with saline. None of these treatments had any effect on female T release (Fig 7.1). The raw data for T release rates are shown in Table 7.1.
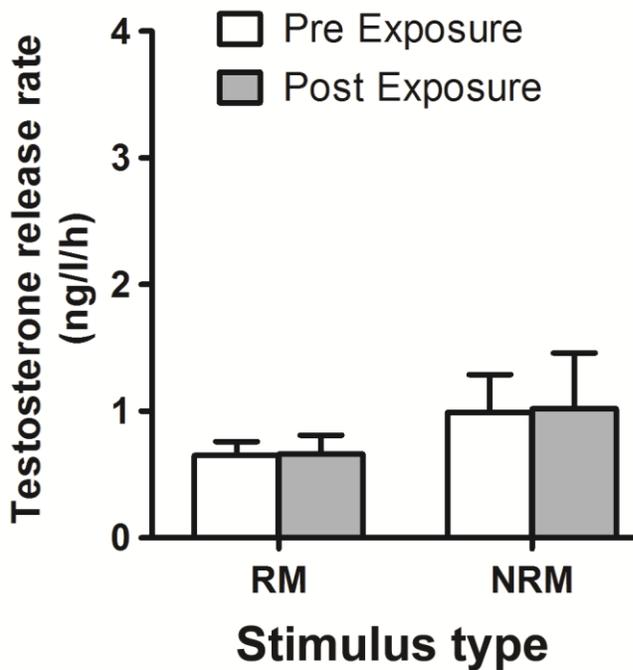
Fig 7.1: Release rate of testosterone (T) measured in female holding water, before and after exposure to male conditioned water. RM = water from reproductive males injected with GnRH. NRM = water from non-reproductive males injected with saline. Neither stimulus led to an increase in the release rate of T as measured in holding water.

Table 7.1: Pre- and post-exposure testosterone (T) release rates for individual females.

Stimulus type	Female #	T release rate (ng/l/hr)		Change
		Pre-exposure	Post-exposure	
RM	1	0.322055	1.33	1.01
	2	0.852774	0.539641	-0.31
	3	0.499536	0.541894	0.04
	4	1.030195	0.366763	-0.66
	5	0.566939	0.531054	-0.04
NRM	6	0.278993	0.177075	-0.10
	7	0.25075	0.305816	0.06
	8	1.039183	0.339101	-0.70
	9	1.392678	1.644765	0.25
	10	1.982625	2.653852	0.67

7.4 Discussion

We were unable, at this time, to find any primer effect of male water on female round gobies. There are several issues to consider.

Following exposure to male conditioned water, we measured levels of T being released by females. Female fish are known to synthesize T, which may increase (as in the flounder, *Pseudopleuronectes americanus*, order Pleuronectiformes, Campbell et al., 1976) or decrease (as in the sea bream, *Pagrus major,*order Perciformes, Matsuyama et al., 1988) prior to ovulation and spawning. Thus, the role of T in female maturation is at least somewhat dependent upon species, and having performed no studies to characterize the endocrine system or production of steroids in the round goby, we have no idea whether T might be low or high prior to ovulation. In addition, there is no evidence that T levels may change in a female fish following exposure to a male fish. Experiments looking at primer effects typically measure gonadotropin levels (e.g. Stacey et al., 1988). In females, it would be best to consider changes in a maturation-inducing hormone such as $17\alpha,20\beta$-P, or better yet, 4-pregnen-17,20β,21-triol-3-one (20β-S), which appears to be the maturation-inducing hormone used by perciformes (Thomas, 1994; King et al., 1997; Pãtino et al., 2001; Sorensen et al., 2005). We measured T due to technical constraints at the time, but T is obviously not an appropriate indicator of male primer effects on females in the round goby.

The next thing to consider is the fact that we chose to measure T levels in water and not in the blood. We use this method mainly for technical reasons: it is very difficult to blood sample the round goby, particularly small, non-reproductive females. Fish this size often simply do not yield enough blood to analyze for steroids and typically the blood sampling is fatal and samples from more than one fish have to be pooled for analysis. Generally, measuring steroids

in water is an acceptable proxy for measuring steroid levels in plasma, and where it has been studied, steroid release rates have a positive relationship with steroid levels in plasma (reviewed by Scott and Ellis, 2007; Scott et al., 2008). Therefore, we can propose that the levels of T measured here are related to the levels of T circulating in NRF round gobies, but there was no increase following exposure to male conditioned water.

In order to assure that the RMs were releasing the steroids which are putative pheromones, we injected these with sGnRHa, which has been shown to increase their release rate of free and substituted forms of 11-O-ETIO (Katare et al., 2011). Therefore, we can assume that the RMs were releasing these steroids. It might be that the four hours post injection was not long enough to allow for the release to reach a peak rate, or that it was not enough time to allow for sufficient amounts of steroids to collect in the conditioned water. When RMs were previously injected with GnRH, 16 hours were allowed to elapse before water collection (Katare et al., 2011). However, following injection with human chorionic gonadotropin, goldfish experience an increase in androstenedione release after only 2 hours (Sorensen et al., 2005) so at least in this case, only 2 hours is sufficient for steroid release. It is also possible that for some reason one – or neither – of the males might not have reacted to the GnRH injection, though the cases of males not reacting to the GnRH injection were rare (Katare, unpublished data). If the experiment is to be repeated, it might be best to use methanol extracted steroids that have already been analysed for 11-O-ETIO immunoreactivity using ELISA. That way, we can expose females to extracts with known levels of 11-O-ETIO. We could have exposed the females to synthetic 11-O-ETIO, but we wanted to make sure we had the best chance to see an effect, so we decided to start by using the water conditioned by the RMs. Ideally, it would be best to start with whole water and if an effect is seen, exposures to individual compounds could follow in order to

determine the exact steroids responsible for any primer effects. Ideally, exposure to one key pheromone could stimulate the same response as exposure to RM conditioned water, which serves as a substitute for exposure to the RM himself. For example, male goldfish exposed to $PGF_{2\alpha}$ treated females (to simulate spawning conditions) release adrostenedione at rates similar to males exposed to just $17\alpha,20\beta$-P (the pre-ovulatory primer pheromone; Sorensen et al., 2005).

In conclusion, though this experiment did not yield any positive results, it was still important for several reasons. We have now established that we can use ELISA to measure released T by females using plates and reagents that we already have in our lab. Should we need to measure release rates of T in the future, we have already used this protocol successfully. Also, we could have been measuring the wrong steroid. For future experiments, the investigator should consider anlyzing the release of different steroids: $17\alpha,20\beta$-P (which we have previously measured in our lab using ELISA, Katare et al., 2011) or 20β-S, which is a maturation-inducing steroid in other teleosts (Thomas, 1994; King et al., 1997; Pãtino et al., 2001; Sorensen et al., 2005), and may be a maturation-inducing steroid in the round goby. Further investigation would help not only to elucidate the possible biological role of 11-O-ETIO release by RM round gobies, but would also provide us with more information about the reproductive and endocrine systems in this very interesting species.

7.5 References

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Chapter 8:

Concluding Remarks

Not enough is known about olfactory biology of fish species other than those which have historically been used as "model" species: goldfish (*Carassius auratus*), zebrafish (*Danio rerio*) and channel catfish (*Ictalurus punctatus*). The work described in this thesis adds to an extensive body of work relating to olfaction in fishes, and identifies and helps to fill gaps in the literature as far as studies on wild fish species and studies using fishes of more diverse taxonomic groups. This thesis has described several studies on the basic olfactory biology of two highly relevant species of fishes. The sea lamprey (*Petromyzon marinus*) and the round goby (*Neogobius melanostomus*) are both invasive species within the Great Lakes. In addition, they occupy vastly different phylogenetic positions; the sea lamprey is an ancient jawless vertebrate of the superclass Agnatha, while the round goby is a member of the diverged fish order Perciformes. Some of the data here, therefore, can be presented in an evolutionary context. Olfactory sensory neurons (OSNs) in fishes display polymorphisms, even in the sea lamprey which has only ciliated OSNs. These three distinct neuronal shapes are seen in fishes as ancestral as the sea lamprey and as diverged as the round goby. The fact that we have seen these polymorphisms in the sea lamprey has highlighted the possibility that OSN polymorphisms could be an evolutionarily conserved feature of the piscine olfactory system. Possibly these polymorphisms respond to separate odors, as seen in teleost fish.

The studies described in this thesis also investigate the olfactory properties of the round goby. Fig 8.1 shows the results of the studies contained in this thesis, using the same flowchart format in Fig 1.3, which outlined the flow of ideas and experiments stemming from Katare et al. (2010). Little olfactory research has focussed on fishes other than the goldfish or the zebrafish, which are both order Cypriniformes, and even fewer studies have used fishes of the order Perciformes, the most speciose fish order. The implication of which is that we don't really know

all differences that may exist in the olfactory system between Cypriniform and Perciform fishes. Previous data, as well as data shown in this thesis, demonstrate that Perciform fishes employ different pheromones than Cyprinids, they use different second messengers to transduce these pheromones, olfactory sensitivities to prostaglandins are different and they may use different prostaglandins than Cyprinid fishes. Studies in this thesis use the round goby to investigate core features of fish olfactory biology including receptor specificity and second messengers, which until now have only been described in a few species.

1 sGnRHa-injected RMs release 11-O-ETIO, 11-O-ETIO-3-sulfate, 11-O-ETIO-17-sulfate, 11-O-ETIO-3-glucuronide and 11-O-ETIO–17-glucuronide.
Urine contains conjugated, but not free 11-O-ETIO (Katare et al., 2010)

2 Female round gobies show an elevated EOG response to methanol extracted isolates from sGnRHa-treated males
Chapter 3

7 Female round gobies have similar EOG responses to RM and NRM urine
Chapter 5

3 Gobies detect 11-O-ETIO &11-O-ETIO-3-s
Chapter 4

4 Round gobies do not detect 11-O-ETIO-3-g or 11-O-ETIO-17-s
Chapter 5

5 Injection with PGF2α led to a non-significant increase in odour response
Chapter 6

6 Exposure to RM water does not increase female T release
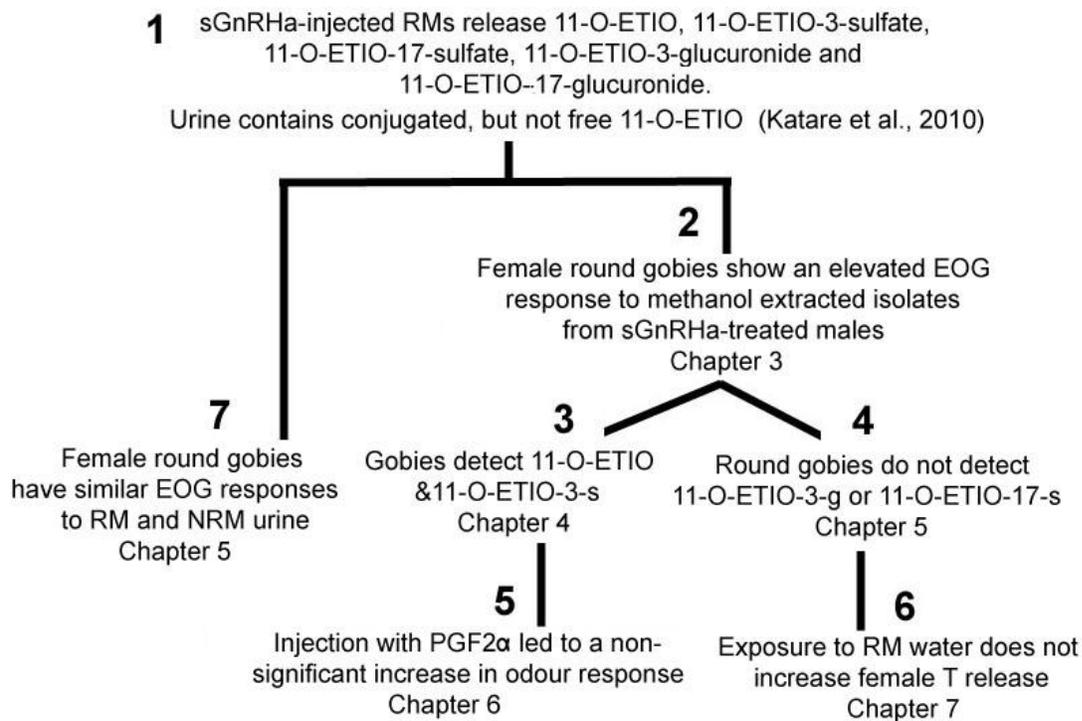Chapter 7

Fig 8.1: Flow chart outlining the results of round goby studies in this thesis, relating to the ideas shown in Fig 1.2. Part 1 is the paper published by Katare et al. (2010) identifying released steroids that are putative pheromones. RM = reproductive male; NRM = non-reproductive male; sGnRHa = salmon gonadotropin releasing hormone analogue.

We had previously shown that injecting reproductive males with salmon gonadotropin releasing hormone analogue (sGnRHa) increases their release rate of putative steroidal pheromones. In conjunction with that, I tested female responses to methanol extracts collected

before and after males were treated with sGnRHa and found that there was an increased female response to the post-injection extract. This is very interesting because it demonstrates how the cue released by the male can change with his reproductive status. This increase in steroids may account for the increased attraction of reproductive female to reproductive male odours and this study is one of the first studies to experimentally demonstrate an increased olfactory response that is associated with a change in endocrine status that has been experimentally induced.

Chapters 4 and 5 describe the olfactory properties of several novel steroids which are released by reproductive male round gobies. Of these, we have identified two (11-O-ETIO and 11-O-ETIO-3-s) which present themselves as putative releaser pheromones based on their potencies, species specificity, receptor specificity and second messengers. We have also eliminated released steroids that – while they may have other biological functions – do not appear to serve as attractive releaser pheromones (e.g. 11-O-ETIO-17-s and others). Experiments in this thesis, as well as current publications from our lab, describe a pheromone system for a Perciform fish; one of only a handful of studies investigating pheromones in this diverse order. Our work, as well as that of others, shows that pheromone systems in the Perciforms have diverged from those of the Cypriniforms. Many fishes of the Cypriniformes appear to share the same pheromonal compounds (i.e. those discovered in the goldfish), while in at least two species of Perciforms studied (the round goby and the Mozambique tilapia, *Oreochromis mossambicus*), putative pheromonal steroids are different from the identified goldfish pheromones.

We have also shown that these putative pheromones act upon separate and specific olfactory receptor mechanisms, indicating that the fish is able to discriminate between these compounds. This indicates these two steroids (11-O-ETIO and 11-O-ETIO-3-s) could be

different pheromones (as opposed to being two components making up one pheromone mixture that requires both elements for a response) eliciting different behavioural responses in the female.

Our study on second messengers in olfactory signal transduction highly similarities and differences in the round goby olfactory system compared to other fishes. For example, the transduction of bile acids via cAMP only appears to be a conserved mechanism in fishes, with only one exception (the Atlantic salmon, *Salmo salar*). Ours is only the second study to show signal transduction mechanisms for steroids (and putative pheromones) and we found that steroids use both second messengers whether they are putative pheromones or general steroids. Ours is the first study to investigate these types of key olfactory mechanisms in a Perciform fish or wild-caught fish.

Our investigation of female olfactory responses to male urine yielded unexpected findings, with non-reproductive male urine inducing larger olfactory responses than the reproductive male urine, but it alerted us to the fact that there may be something in the urine of non-reproductive males that we have not yet identified, that accounts for its olfactory potency. This study showed that using male urine as an odour source may not be the best choice for EOG or behavioural studies, as it elicited unpredictable responses from the non-reproductive females and we cannot account for all of the components of the urine.

In addition to these studies on the olfactory properties of OSNs and released steroids, I have performed pilot experiments involving other aspects of round goby olfactory biology. Chapter 6 is an initial investigation into the possibility of modulating olfactory responses via prostaglandins. There have been no similar studies described in any other female fish species, and if this experiment is successfully completed, it would be extremely novel.

We have developed a protocol for evaluating the possible male primer pheromone effects on female round gobies. This protocol is described in chapter 7. Although we saw no effect, we have established, at least, that we can measure testosterone in the water, and the protocol is easily adapted to the measurement of other steroids. Even the measurement of testosterone is interesting, as we currently have no idea about natural steroid levels in the female round goby. The ELISA assay is straightforward to perform and the exposure protocol is simple; thus, this experiment presents itself as an easy and quick project, suitable for an undergraduate. The ability to identify primer pheromones in the round goby may assist us in further study, if we can help induce the females to produce eggs and reach reproductive status by exposing them to male odours.

Overall, this thesis has made significant contributions to the field of olfactory biology by investigating olfactory sensory neurons in two diverse species of fishes occupying diverse phylogenetic positioning. Our studies of the round goby highlight differences between a wild species and the previously studied fish species that have been bred in the lab over many generations. Very few studies have looked at pheromones other than established goldfish pheromones in any species of fish, and ours is one of only two (to my knowledge) that have identified novel substances that are putative pheromones in a fish species. Our work is also of note due to the round goby's status as an invasive species in the Great Lakes. The eventual establishment of a pheromone trapping biocontrol method could prove highly advantageous and beneficial to the future health and ecology of the lakes. Additionally, a better understanding of the reproductive endocrinology of this species would make future lab studies more productive. In conclusion, it is our belief that the work presented here has made a significant and unique contribution to the fields of olfactory biology and chemical ecology.

Appendix A

List of Reagents

ITEM	SUPPLIER	CATALOGUE #	BATCH/LOT #
Steroids			
11-O-ETIO	Steraloids	A3460-000	Multiple
11-O-ETIO-3-s	Steraloids	A3500-000	Multiple
11-O-ETIO-17-s	Steraloids	A3232-000	Multiple
11-O-ETIO-3-g	Steraloids	A3232-000	B0791
ETIO	Steraloids	A3610-000	Multiple
Estrone	Steraloids	E2300-000	B0294
Cortisol	Steraloids	Q3880-000	Unknown
Cortisone	Steraloids	Q2500-000	Unknown
$17\alpha,20\beta$-P	Sigma-Aldrich	P6285	Unknown
Pharmacological Agents			
Diltiazem	Calbiochem	309866	D00046189
Forskolin	Calbiochem	344270	D00069272
	Sigma-Aldrich	F6886	126K5063
	Tocris	1099	3A/99397
SQ, 22 536	Calbiochem	568500	D00066942
	Enzo Life Sciences	BML-CN140	3-W5184k
	Sigma-Aldrich	S153	116K4072
U73122	Calbiochem	662035	D00051672 & 84502
	Enzo Life Sciences	BML-ST391	3-W5104g
	Sigma-Aldrich	U6756	027K46141
Miscellaneous			
L-alanine	Sigma-Aldrich	S4500	117K0835
Taurocholic Acid	Sigma-Aldrich	T4009	087K0745
Lutalyse	Vet purchasing co ltd	Unknown	Unknown

Appendix B

Raw data

Responses to 3α,17β-dihydroxy-5β-androstan-11-one (1D)

FISH ID	10^{-5} M Ala	MOLAR CONCENTRATION				
		-8	**-9**	**-10**	**-11**	**-12**
F06-08-09	1.38	0.52	0.48			
		0.27	0.00			
		0.60	0.00			
F08-11-09	2.20	0.75	1.25	0.67	0.84	0.00
		1.82	0.96	0.63	0.00	0.00
		1.80	1.16	0.54	0.00	0.00
F08-12-09	2.96	0.00	1.10	0.00	0.00	
		0.00	1.21	0.00	0.00	
		0.00	0.68	0.00		
F08-14-09	1.12	0.00	0.00	0.00	0.00	0.00
		0.00	0.00	0.00		
		0.00	0.00	0.00		
F08-14-09	2.32	0.00	0.00	0.00		
		0.00	0.00	0.00		
		0.00	0.00	0.00		

Methanol extracted isolates used for analysis

Extract Codes	Female tested on (EOG)
sGnRHa Treated **Both pre-and post-treatment**	
16/7/2007 G1	G068, G072, G073
16/7/2007 G2	G072, G073, G077
16/7/2007 G3	G068, G073, G077
18/7/2007 G1	G084
18/7/2007 G3	G089
23/5/2007 G2	G087
Saline Treated **Both pre-and post-treatment**	
16/7/2007 S 18/7/2007 S 23/7/2007 S	G068, G072, G073, G077, G084, G089 G087

Appendix C

Mass spectra of steroid odours
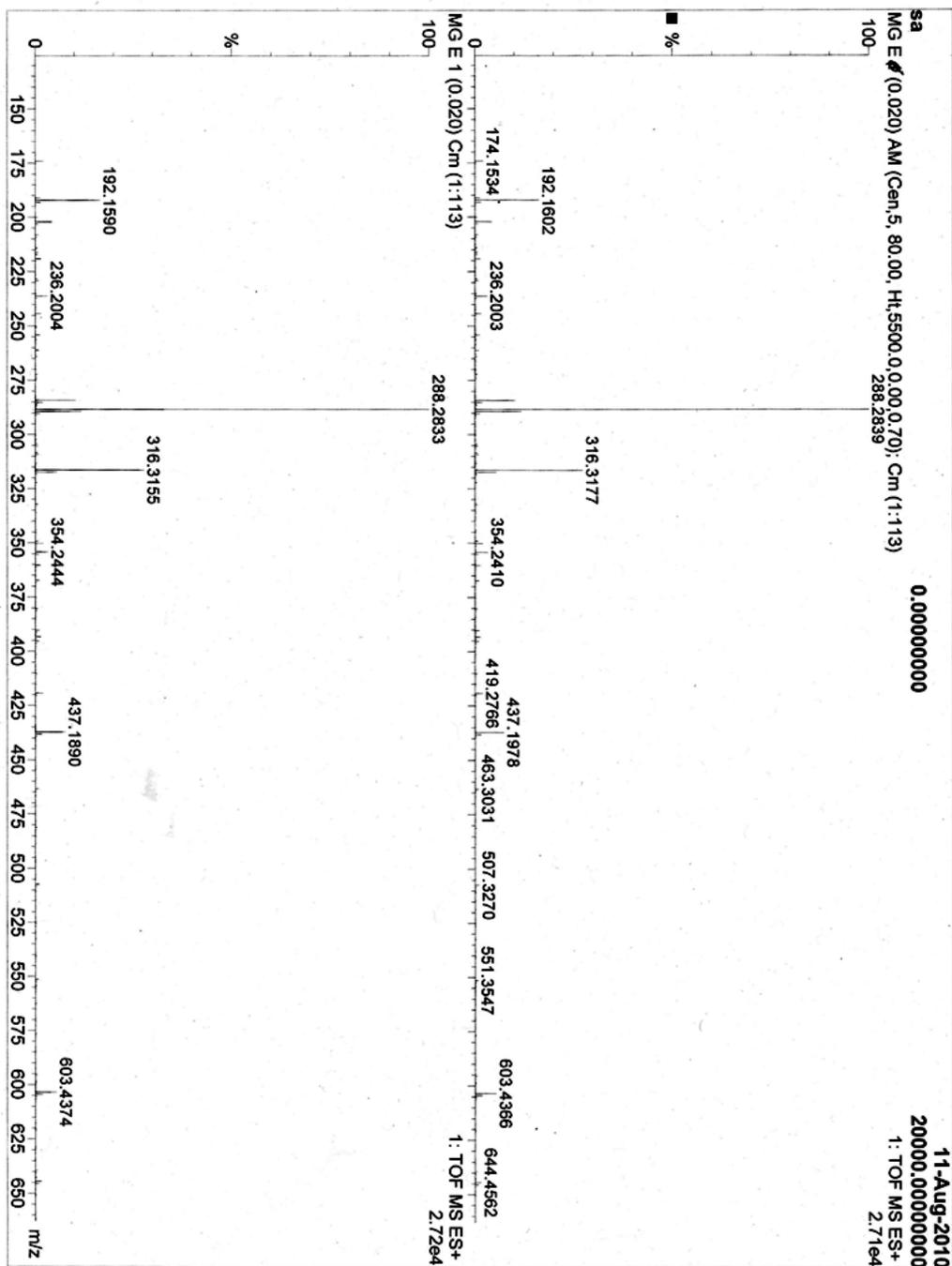
Mass spectra for 11-O-ETIO-3-g steroid stocks used for EOG testing.

Expected peak is 383, but the mass spectra for two different steroid stock solutions run show no peak at 383, but large peaks at 192, 288 and a smaller peak at 316, indicating that there is some contamination and that the compound my have broken down.
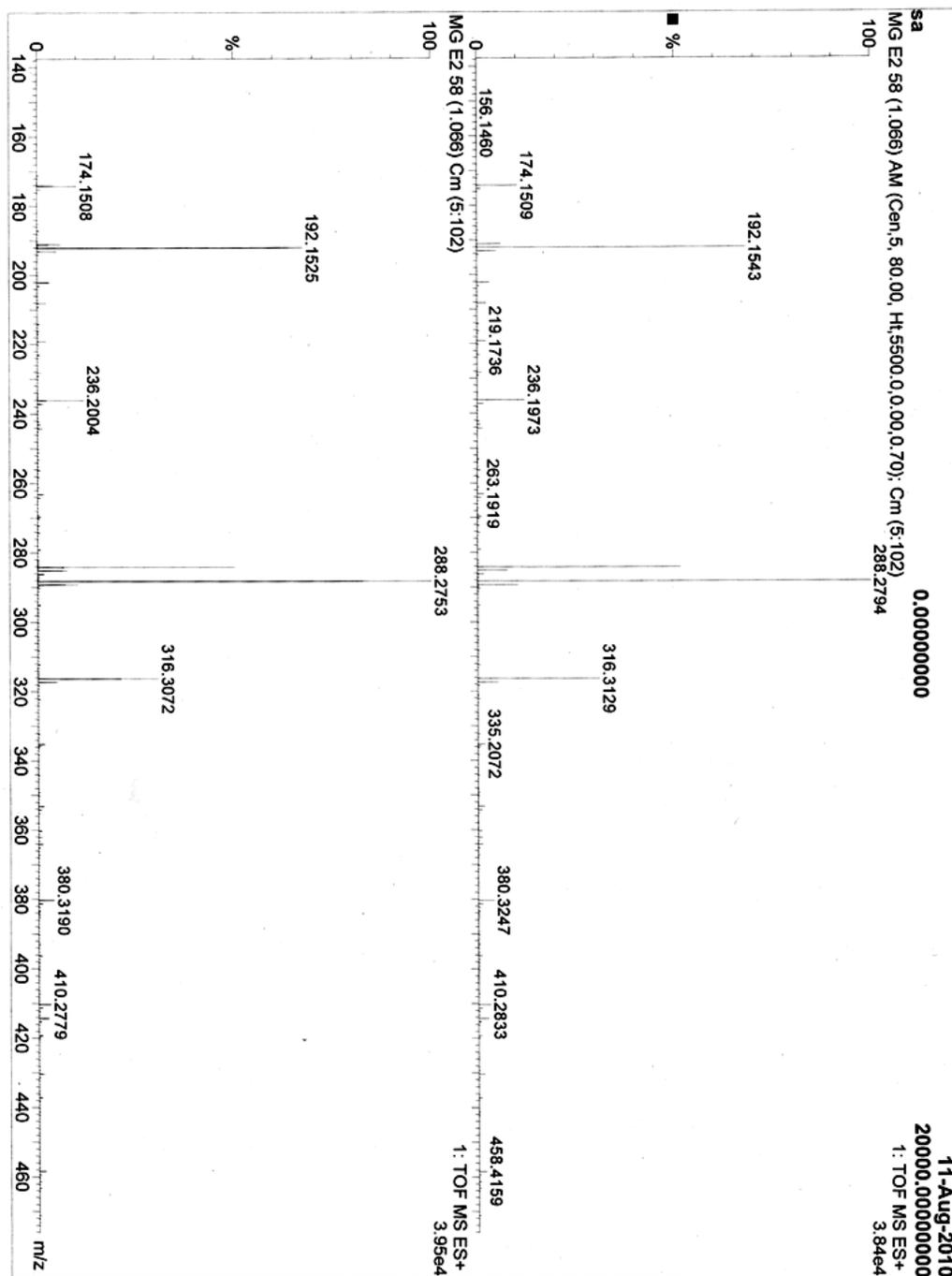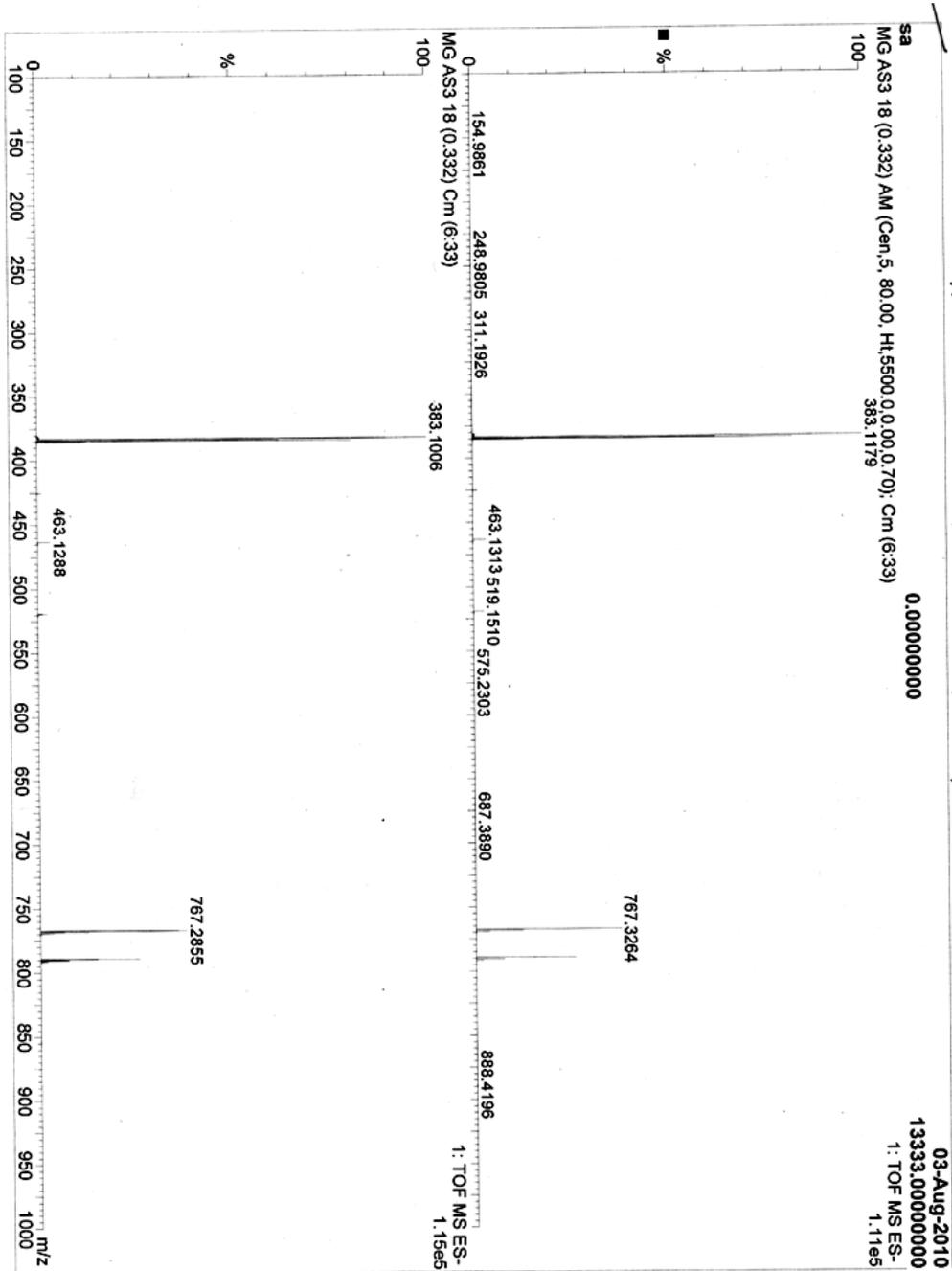
Appendix C

Mass spectra of steroid odours

Mass spectra for 11-O-ETIO-3-g steroid stocks used for EOG testing.

Expected peak is 383, but the mass spectra for two different steroid stock solutions run show no peak at 383, but large peaks at 192, 288 and a smaller peak at 316, indicating that there is some contamination and that the compound my have broken down.

Stock #2

Appendix C

Mass spectra of steroid odours

Mass spectra for 11-O-ETIO-3-s steroid stock used for EOG testing.

The expected peak for 11-O-ETIO-3-s is at 383 as shown here. Run as positive control during MS run for 11-O-ETIO-3-g.

Appendix D

Amount of immunoreactive substituted 11-O-ETIO as measured by ELISA in the urine samples tested for EOG responses.

The starting volume of urine used for EOG testing was standardized so that the volume used represents 1/10th of the total volume of urine collected after 4 hours. The amount of substituted 11-O-ETIO is the amount (in ng) contained in the pure urine before dilution for EOG testing. The estimated [substituted 11-O-ETIO] in the 100x dilution tested on females by EOG is the estimated molarity of substituted 11-O-ETIO in the 100x dilution; this estimation was based on the molecular weight of the most abundant conjugate in the urine, 11-O-ETIO-17-s, which is 385 kDa.
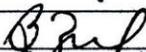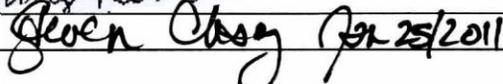
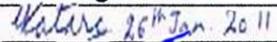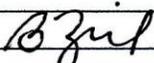|	Urine code	Total urine vol (µl)	Amt substituted 11-O-ETIO in the EOG volume (ng)	Est. substituted 11-O-ETIO in the 100x dilution tested by EOG (X 10^{-10} M)	Avg EOG response (mV)	Female GSI (%)	Female code (EOG)
RM	18	8.8	0.53265194	1.57	0.91	1.37	AL2008-09
	38	73.8	25.35975378	8.89	2.11 0.40	6.48 4.02	AL2008-05 AL2008-06
	58	8.8	0.38906622	1.15	1.71	5.51	AL2008-07
	165	29	0.93945993	0.841	1.00 1.35	0.57 0.79	AL2008-17 AL2008-20
NRM	107	39.8	0.24140638	0.157	4.93 1.01	6.48 4.02	AL2008-05 AL2008-06
	114	23.3	0.1048706	0.117	3.17	5.51	AL2008-07
	115	16.3	0.01419166	0.0226	0.48	1.37	AL2008-09
	133	27.9	0.06933814	0.0646	0.30 0.47	0.57 0.79	AL2008-17 AL2008-20

Appendix E

Written permission from co-authors to include content which is the result of collaborations in this thesis

I, the undersigned, do state that I give my permission for Alyson J. Laframboise to include in her thesis, work which represents the combined efforts of myself and Ms. Laframboise (as well as listed co-authors), the details of which are listed below.

Thesis Chapter 2 – Olfactory sensory neurons in the sea lamprey display polymorphisms
Published 2007 in Neuroscience Letters 414(3);277-281

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Barbara Zielinski	
Xiang Ren	
Steven Chang	Jan 25/2011

Thesis Chapter 3 – The effect of elevated steroids released by reproductive male round gobies (*Neogobius melanostomus*) on olfactory responses in females
Accepted to the Journal of Chemical Ecology, Jan 2011

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Barbara Zielinski	
Alexander Scott	

Thesis Chapter 4 – Female round gobies (*Neogobius melanostomus)* detect and discriminate between steroids released by male round gobies
Submitted to the Journal of Experimental Biology, Nov 5th, 2010

Co-Author Name	Co-Author Signature
Barbara Zielinski	
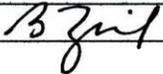
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