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Female Round Goby (Neogobius Melanostomus) Behavioural Responses to Fractionated Male-Conditioned Water and to Steroids Released by Males

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by
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ABSTRACT

The round goby, *Neogobius melanostomus,* is an invasive fish species to the Laurentian Great Lakes. There is evidence that male round gobies release steroids that may function as pheromones. We have developed a novel high-throughput behaviour assay for testing the attractiveness of odours to female round gobies in order to progress towards the identification of pheromones. Both reproductive (RF) and non-reproductive (NRF) females were attracted to a blend of synthetic steroids. Both RFs and NRFs were strongly attracted to urine and methanol extracts of urine from GnRH-injected males. An HPLC fraction pool taken from GnRH-injected male-conditioned water including unknown conjugate(s) of 11-oxo-ETIO was highly attractive to RFs and less attractive to NRFs. In contrast, an HPLC fraction pool including free 11-oxo-ETIO was attractive to NRFs - but not to RFs. Our findings suggest that males release unknown conjugate(s) of 11-oxo-ETIO into the urine that may function as reproductive pheromones.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

2-PE – 2-phenoxy ethanol
11β-OH-Ad – 11β-hydroxyandrostenedione
11-KT – 11-ketotestosterone
11-oxo-ETIO – 3α-hydroxy-5β-androstan-11,17-dione; 11-oxo-etiocholanolone
11-oxo-ETIO-g – 11-oxo-etiocholanolone-glucuronide
11-oxo-ETIO-s – 11-oxo-etiocholanolone-sulfate
Ad – androstenedione
ANOVA – analysis of variance
ACN – acetonitrile
cAMP – cyclic AMP
ELISA – enzyme-linked immunosorbent assay
EOG – electro-olfactogram
ETIO – 5β-androstan-3α-ol-17-one; etiocholanolone
ETIO-g – etiocholanolone-glucuronide
ETIO-s – etiocholanolone-sulfate
$G_{q/11}$ – a microvillous olfactory sensory neuron-specific G protein
G_{olf} – a ciliated olfactory sensory neuron-specific G protein
G_o – a crypt olfactory sensory neuron cell-specific G protein
GnRH – gonadotropic releasing hormone – used to increase release of steroids
s-GnRH – salmon GnRH analogue
GSI – gonadosomatic index – gonad weight / total body weight
HPLC – high performance liquid chromatography
L-ala – L-alanine
NRF – non-reproductive female
NRM – non-reproductive male
M – Molar (moles/L)
MS-222 – tricaine methane sulfonate
ORN – canonical odourant receptors
PGF – F-series prostaglandins
$PGF_{2\alpha}$ – prostaglandin $F_{2\alpha}$
RF – reproductive female
RM – reproductive male
SEM – standard error of the mean
T – testosterone
TFA – trifluoroacetic acid
V1R & V2R – vomeronasal-type odourant receptors
Vent/min – gill ventilations per minute

1 INTRODUCTION

1.1 COMMUNICATION & PHEROMONES

Many animals emit chemical signals to communicate with members of the same species or members of different species. According to Law & Regnier (1971), substances that are externally secreted by an individual (the sender) and detected by the olfactory system of a second conspecific individual (the receiver) are known as pheromones. Upon detection, pheromones may release specific reactions, such as a specific behaviour or internal hormonal responses. Pheromones may act in minute amounts, are generally species-specific, and tend to have limited molecular overlap between closely related species

In many animals, a subset of pheromones, known as reproductive pheromones, are used to facilitate communication between males and females. These pheromones may be involved in mate selection, same-sex aggression, and facilitating gametogenesis. According to Sorensen & Stacey (1999), a reproductive pheromone evokes a specific and adaptive reproductive response in conspecifics. This response does not require specific learning.

1.1.1 Olfaction in Fishes: an Overview

The olfactory system in fishes includes a peripheral olfactory organ lined with olfactory epithelium (reviewed in Trotier & Dϕving, 1996; Zielinski & Hara, 2007). Neuronal cells located within this epithelium contain receptors for odourant molecules. Some of these receptors are G-protein-cAMP-linked membrane receptor proteins that are specific to different odourants. During olfactory sensory transduction, cAMP-gated sodium and calcium channels are opened and depolarization occurs following ionic flux through channels. If the cell is depolarized past its firing threshold, it fires an action potential that propagates along the axon to the axon terminals in the olfactory bulb, located within the brain.

Canonical ciliated odourant receptor neurons (ORNs) involve activation the G_{olf} protein, which increases intracellular cAMP levels (Ngai *et al.*, 1993a). There are, however, other types of sensory neurons. For example, in the channel catfish, the transduction cascade of microvillous ORNs include the G protein $G_{q/11,}$ and crypt cell ORNs contain G_o (Hansen *et al.*, 2003). In addition teleost olfactory sensory neurons contain V1R and V2R receptors, the class of receptors originally found in vomeronasal organs of mammals (reviewed in Hansen & Zielinski, 2005).

There are several classes of odourant molecules detected by fishes, and different classes are detected by different receptor cells in the epithelium (Ngai *et al.*, 1993b), which may lead to spatial mapping in brain structures, such as the olfactory bulb (Nikonov & Caprio, 2004; Rolen & Caprio, 2007). For example, Koide *et al.* (2009), demonstrated that in zebrafish, there are specific subsets of microvillous olfactory sensory neurons that project to the lateral region of the olfactory bulb that are important for amino acid-mediated feeding behaviour.

Thus, it is important to understand the different classes of odourant molecules that are detected be fishes.

1.1.2 Odourants in the Aquatic Environment

Several classes of odourant molecules that exist in aquatic environments are detected by various fishes. According to Sorensen & Caprio (1999), there are four main classes of odourants detected by fishes: amino acids, bile acids, prostaglandins, and sex steroids. Liberles & Buck (2006) discovered that fish possess a class of odourant receptors that detect amines.

i. Amino Acids

Amino acids are food odours and tend to function primarily as feeding cues (Hara, 1992), and may actually release feeding behaviours in some species (Hara, 2006a). For example, L-alanine (**Figure 1**) and L-serine elicit feeding behaviours in the striped bass, *Morone saxatilis* (Papatryphon & Soares, 2000). Naïve zebrafish show increased swimming activity associated with feeding behaviour when presented with L-alanine and L-valine (Braubach *et al.*, 2009).

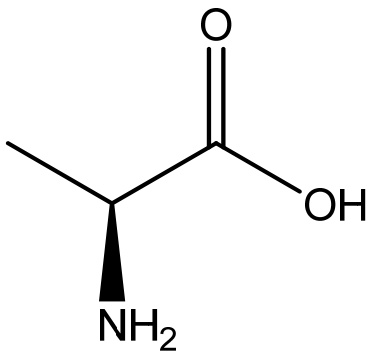

Figure 1. Chemical structure of the amino acid L-alanine.

ii. Bile Acids

Bile acids, such as taurocholic acid (**Figure 2**), are also odours detected by fishes (Sorensen & Caprio, 1999). Bile acids are used by the digestive system of vertebrates, where they aid in the digestion of fats (Silverthorn, 2004). Bile acids are formed in the liver from cholesterol, move into the intestinal lumen where they are taken back up into the liver through intestinal uptake, or are released into the external environment through the feces. Recently, it has discovered that bile alcohols (derived from bile acids) released by male sea lampreys (*Petromyzon marinus*) act as sex (Li *et al.*, 2002) and migratory (Sorensen *et al.*, 2005a) pheromones.

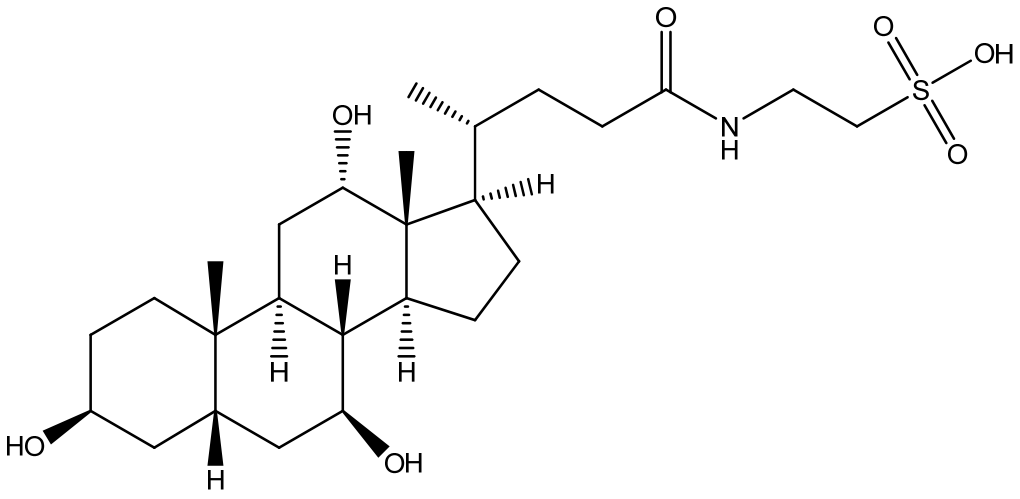

Figure 2. Chemical structure the bile acid taurocholic acid.

iii. Prostaglandins

Prostaglandins are lipid-derived molecules that play a role in mediating various physiological processes in animals including inflammatory and pain responses, vascular smooth muscle tone, and hormonal control of reproduction

(Silverthorn, 2004). In the goldfish (*Carassius auratus*), it has been found that gravid females release F-type prostaglandins (PGFs), such as $PGF_{2\alpha}$ (**Figure 3**), that induce spawning behaviours in males of all stages (Kobayashi *et al.*, 2002). In addition, injections of prostaglandins have been able to induce spawning behaviours in both reproductive and non-reproductive female cichlids, *Cichlasoma bimaculatum*, independently of ovarian hormone influence (Cole & Stacey, 1984). Prostaglandins are commonly released by reproductive females and detected by reproductive and non-reproductive males in Cypriniform fishes, including goldfish (Sorensen *et al.*, 1993), crucian carp (*Carassius carassius*) (Bjerselius & Olsen, 1993), and the common carp (*Cyprinus carpio*) (Irvine & Sorensen, 1995). Prostaglandins clearly play a role in pheromonal communication in these species. However, the olfactory epithelium of male and female round gobies (*Neogobius melanostomus*, Perciformes: Gobiidae), does not respond to prostaglandins (Murphy *et al.*, 2001).

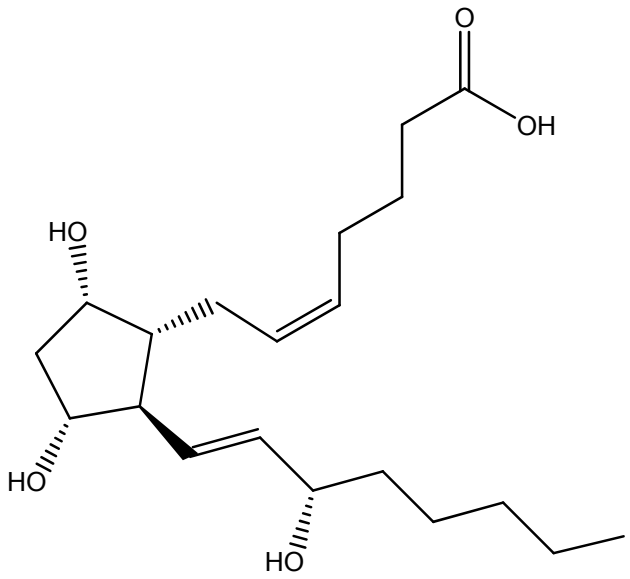

Figure 3. Chemical structure of prostaglandin $F_{2\alpha}$.

iv. Sex Steroids

Sex steroids are derivatives of a common skeleton ring structure. Androgens and estrogens are C_{19} compounds, whereas progestogens are C_{21} steroids (Fox & Whitesell, 2004). These base molecules may be conjugated with various groups at specific carbons around the rings (**Figure 4**). Conjugated groups that are below the plane of the ring are referred to as α; groups that are above the plane are referred to as β.

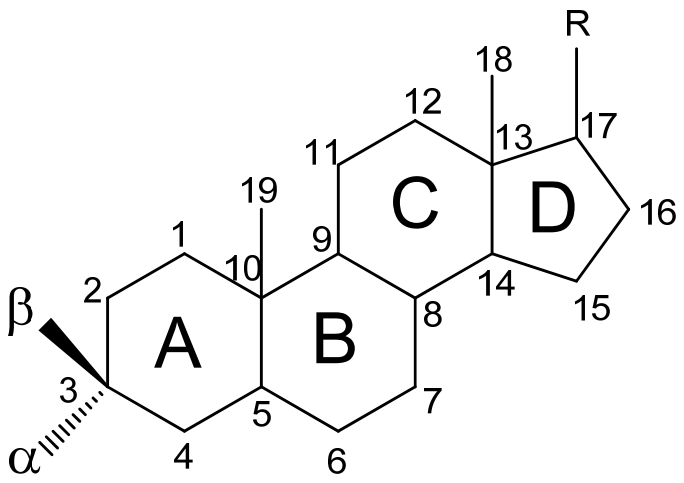

Figure 4. The steroid skeleton ring with corresponding carbon numbering and nomenclature system. Each ring is assigned a different letter, and conjugates may occur at any of the numbered carbons (R). Conjugates that lie below the plane of the ring are referred to as α; conjugates that lie above the plane are referred to as β.

The release of sex steroids that are synthesized in the testes of fish may take one of several main routes: the gills (the main route for free steroids), the urine (the main route for sulfated steroids), or the feces (the main route for glucuronides) (Scott & Vermeirssen, 1994; Vermeirssen & Scott, 1996). Conjugation can occur in various organs, such as the testes, the seminal vesicles,

or the liver (Scott & Vermeirssen, 1994). There has also been evidence of steroids (both free and conjugated) released in the seminal fluids, directly from the seminal vesicles (Schoonen *et al.*, 1987), and the milt (Scott & Vermeirssen, 1994). There is no evidence, however, that the release of steroids in the milt facilitates spawning between males and females; the milt of black gobies, *Gobius jozo* (Perciformes: Gobiidae) may attract subordinate males into the nests of spawning males (Locatello *et al.*, 2002).

Several species of fishes use steroids as reproductive pheromones. The use of steroid pheromones is seen in the European eel (*Anguilla anguilla*, Anguilliformes: Anguillidae). The gonadal development of the immature male eels was stimulated when these fish were kept in proximity to salmon pituitary extract injected males (to stimulate the hypothalamic-gonadal axis) that released elevated levels of testosterone and 11-ketotestosterone (11-KT) (Huertas *et al.*, 2006). In goldfish, males release androstenedione (Ad) in the presence of reproductive and non-reproductive females to attract females and induce reproductive females to spawn (Sorensen *et al.*, 2005b; Sisler & Sorensen, 2008).

In male Perciform fishes, reproductive steroids are typically 5α and 5β reduced androgens. For example, the testes in male black gobies, *Gobius jozo*, readily synthesize 5β reduced androgen metabolites, such as 5β-androstan-3α-ol-17-one (etiocholanolone, ETIO, **Figure 5A**) (Colombo *et al.*, 1977; 1980), whereas the testes in the urohaze goby, *Glossogobius olivaceus*, synthesize 5α reduced androgens, such as 5α-pregnane-3,20-dione (Asahina *et al.*, 1985). In the case of the black goby, males release significant amounts of etiocholanolone glucuronide

(ETIO-g, **Figure 5B**), which has been shown to strongly attract gravid females (Colombo *et al.*, 1980).

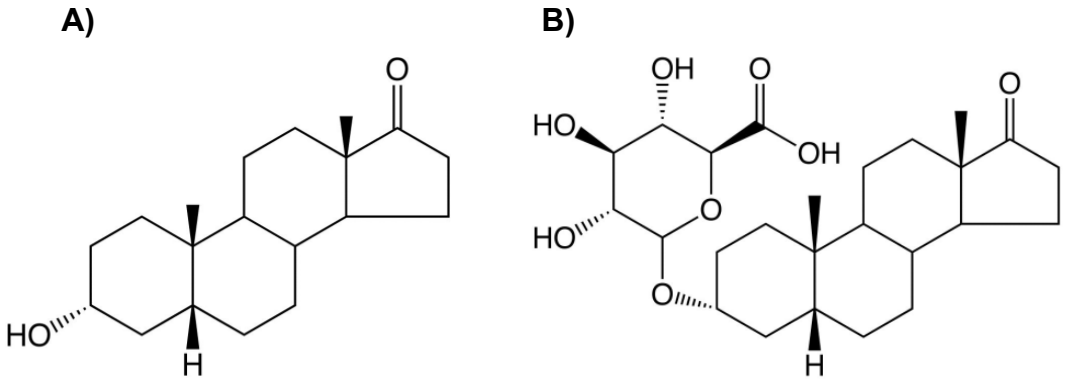

Figure 5. Chemical structures of etiocholanolone (ETIO) (**A**) and etiocholanolone-glucuronide (ETIO-g) (**B**).

In contrast, the testes of male round gobies do not produce ETIO-g, and synthesize minor quantities of ETIO and ETIO-s compared to the black goby (Arbuckle *et al.*, 2005). However, male round gobies synthesize 11-oxo-ETIO (**Figure 6**) in the testes (Arbuckle *et al.*, 2005) and seminal vesicles (Jasra *et al.*, 2007); these were not found in the black goby (Colombo *et al.*, 1977). There is also evidence that male round gobies release 11-oxo-ETIO and conjugates into the water (Kereliuk, *et al.*, 2009; Katare *et al.*, in preparation). In order for a steroid to function as a pheromone, it must be released into the water, and elicit a response from the intended receivers. The present study will focus on extracts of male-conditioned water that contain putative pheromonal compounds, including 11-oxo-ETIO and its conjugates. A full discussion on progress towards the identification of sex pheromones in the round goby can be found in section 1.3.2.

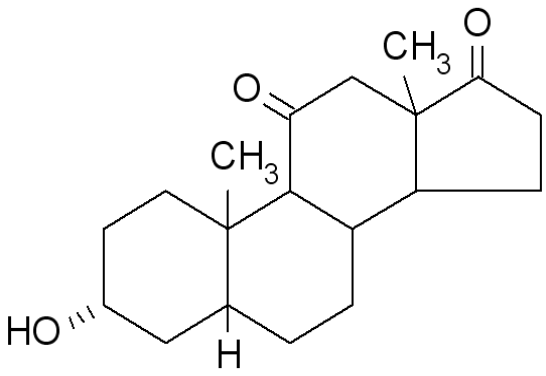

Figure 6. Chemical structure of 11-oxo-etiocholanolone (11-oxo-ETIO).

1.2 GILL VENTILATION & REPRODUCTIVE BEHAVIOURS IN FISHES

1.2.1 Gill Ventilation

In teleost fish, rhythmic movement of the opercular flaps allows for water flow over the gills, which is essential for allowing gas exchange to occur across the gills (Diana, 2004). In most teleosts, gill ventilation rates increase in low oxygen environments in order to move water over the gills at a faster rate. However, gill ventilation rates may also be linked to olfaction, with fishes increasing gill ventilation rates in order to facilitate odour detection. For example, Nevitt *et al.* (1991) found that flounders use quick increases in gill ventilation, perhaps analogous to sniffing in mammals, in the presence of food odours. Thus, while an increase in gill ventilation may not be a direct indication that a given odour is attractive to a fish, it suggests that there is an olfactory-driven behavioural response to that particular odour.

Several gill ventilation studies have been performed in the round goby, as well. Male round gobies, for example, increased gill ventilation rates significantly when presented with estrone (1,3,5(10)-estratrien-3-ol-17-one), which is released by female round gobies (Belanger *et al.*, 2006; Belanger *et al.*, 2007). This gill ventilation response is mediated by olfaction, as there were no changes in gill ventilation rates during nasal occlusion or following copper-induced degeneration of olfactory sensory neurons (Belanger *et al.*, 2006). Furthermore, females increased gill ventilation rates when exposed to estrone as well as several other steroids, including ETIO and 11-oxo-ETIO (Murphy *et al.*, 2001).

1.2.2 Mate Choice & Spawning Behaviours

There are several differing reproductive behaviours found in fishes. For example, female rainbow trout, *Oncorhynchus mykiss* (Salmoniformes: Salmonidae), select sites in high flow streams, where they deposit their eggs. Males compete for the right to fertilize the eggs, and then abandon the eggs (Hartman, 1970). In contrast, male bluegills, *Lepomis macrochirus* (Perciformes: Centrarchidae), build benthic nests in shallow areas (Gross, 1991). The males guard the nests against other males, and each male will try to attract as many females as possible to his nest to lay eggs, which the male fertilizes. These males are known as parental males. Two other male behaviour profiles exist in bluegills: mimics, which attempt to mimic females in order to gain access to another male's nest to fertilize his eggs, and satellites, which mature at small sizes and dart in and fertilize eggs at critical moments in the spawning of other males and females.

Regardless of which sex attracts the other for spawning, there are certain characteristics specific to each species that determines whether an individual is attractive to the opposite sex. For example, the male peacock blenny, *Salaria pavo* (Perciformes: Blenniidae), has a bright yellow helmet of adipose and connective tissue on the top of his head with a dark bar running parallel to the head profile through the eyes to emphasize the helmet (Patzner, 2008). This helmet functions as a signal and may be involved in mate selection.

Other fishes may use pheromones to attract members of the opposite sex prior to spawning. For example, female goldfish release $PGF_{2\alpha}$ into the water, and males are attracted to females and perform spawning behaviours, such as chasing (Sorensen *et al.*, 1988; Kobayashi *et al.*, 2002; Stacey & Sorensen, 2002). Likewise, reproductive male goldfish release androstenedione (Ad) into the water, which attracts females and induces spawning behaviours (Sorensen *et al.*, 2005b). The gills of male sea lampreys release a bile acid sex pheromone, 7α,12α,24-trihydroxy-5α-cholan-3-one-24-sulfate, that has been shown to act as a potent attractant for females (Li *et al.*, 2002). An early study in the frillfin goby, (*Bathygobius soporator*) (Perciformes: Gobiidae) showed that males exposed to water that had been conditioned by gravid females were induced to court, but exposure to immature females did not stimulate this response (Tavolga, 1956). More recently, Colombo *et al.* (1977; 1979; 1980) found a similar trend in black gobies, a benthic species with nest-guarding males. This reproductive status-specific behaviour may be an evolutionary mechanism that allows only individuals that are imminently ready to spawn to find each other using pheromones.

There have been studies in the Mozambique tilapia (*Oreochromis mossambicus*, Perciformes: Cichlidae) that focus on urine released by dominant males. Mozambique tilapia are polygynandrous; males defend a lek-style spawning territory that is visited by females (Bruton & Boltt, 1975). In this species, dominant males store large amounts of urine that they release in pulses in the presence of receptive pre-ovulatory females, but not in the presence of post-ovulatory females (Barata *et al.*, 2007). Urine from dominant males elicited more potent olfactory epithelial responses in females than in subordinate males (Barata *et al.*, 2007) and a sterol-like odourant was found in much higher quantities in the urine of dominant males than in the urine of subordinate males, suggesting that this unknown compound may act as a pheromone signaling dominance in this species (Barata *et al.*, 2008).

A previous assay of the attractiveness of reproductive male urine to female round gobies found that females spent more time at an artificial nest when reproductive male urine was delivered from the nest than when non-reproductive male urine was delivered (Yavno & Corkum, in preparation). Meunier & Corkum (2007) found that reproductive males, but not non-reproductive males, increased the number of urination pulses in the presence of reproductive and non-reproductive females. These previous studies support the idea that round goby males use urine as a vehicle for dispersing reproductive pheromones that attract females to nests.

1.2.3 Studying Reproductive Behaviour & Pheromonal Communication in Fishes: the Need for a High-Throughput Method

The existence of such attraction behaviours in response to pheromones has led to the development of lab assays that use delivery of odourants into a tank to determine whether or not odourants are attractive. This approach has been used for the common carp (Sisler & Sorensen, 2008), goldfish (Kobayashi *et al.*, 2002; Sisler & Sorensen, 2008), fathead minnow, *Pimephales promelas* (Cyprinformes: Cyprinidae) (Cole & Smith, 1992), peacock blenny (Serrano *et al.*, 2008), zebrafish (Braubach *et al.*, 2009; Koide *et al.*, 2009), and the round goby (Belanger *et al.*, 2004; Gammon *et al.*, 2005; Corkum *et al.*, 2008). All of these studies involved delivering an odour, such as a putative pheromone, into a tank. Fish that swam towards odour or spent more time near the odour source were said to be attracted to the particular odour of interest.

In this thesis, a combination of swimming activity analysis and gill ventilation rate analysis, adapted from these previous studies, assays attraction behaviours in female round gobies to urine from GnRH-injected males and fractionated male-conditioned water. One key difference between the present study and previous behavioural studies is that a high-throughput system is used, which allows five fish in separate tanks to be analyzed at one time. The relatively small size of the tanks allows for the delivery of relatively small amounts of odour to five fish at once, whereas previous studies only tested one fish at a time. The one-at-a-time approach is quite time consuming; here we were able to test greater numbers of fishes in a shorter period of time. This advantage is important given the time constraints of the seasonality of the reproductive season. Since most of

the test odours were prepared directly from biochemical extracts of male round gobies excretions, it was important to be able to obtain responses to small amounts of test solutions. We hope to use this approach to progress towards the identification of the reproductive pheromones used by the round goby.

1.3 THE ROUND GOBY

1.3.1 Biology of the Round Goby

The round goby, *Neogobius melanostomus* (Perciformes: Gobiidae) (**Figure 7**), is an invasive species to the Laurentian Great Lakes that was likely brought over from the Ponto-Caspian region of Eastern Europe in ship ballast water released into the St. Clair River at some point prior to 1990 (Jude *et al.*, 1992). Within five years, round gobies had spread throughout the five Great Lakes (Jude, 1997). Round gobies are now spreading to inland lakes, such as Lake Simcoe (Wegman, 2006).

While most native species in the Great Lakes spawn only once per reproductive season, female round gobies are multiple spawners (up to once every 20 days) with an extended reproductive season (Corkum *et al.*, 1998), a 95% fertilization rate, and a 95% hatching rate, with parental care contributing to the success of offspring (MacInnis & Corkum, 2000). Parental males aggressively defend dark cavity nests, in which females deposit eggs during the spawning season (Charlebois *et al.*, 2001). Male round gobies defend preferred rocky nesting sites against predation, and often eat the eggs and young of native fishes, such as lake trout (*Salvelinus namaycush*) (Fitzsimons *et al.*, 2007), lake sturgeon (*Acipenser fulvescens*, listed as a species of concern by the Species Risk Act) (Nichols *et al.*, 2003), and smallmouth bass (*Micropterus dolomieu*) (Steinhart *et al.*, 2004). As a result, gobies monopolize preferred spawning habitats of native fish (Jude, 1997; Charlebois *et al.*, 2001).

It has been suggested that the aggressive reproductive tactics of the round goby may be partly responsible for the decline of benthic species such as

the mottled sculpin (*Cottus bairdi*) (Dubs & Corkum, 1996). In a recent study of fish distribution in the shoreline areas of the lower Great Lakes, only a handful of native species, such as the mottled sculpin and logperch (*Percina caprodes*), were collected, whereas an abundance of round gobies was collected from all of these sample sites (Dopazo *et al.*, 2008).

Round gobies have also been implicated as a vector in a recent outbreak of avian botulism. Yule *et al.* (2006a; 2006b) found that round gobies were able to withstand repeated low-level exposures to *Clostridium botulinum* type E neurotoxin, which may persist and cause mortality in fish-eating birds.

There have been several relatively costly and ineffective attempts to control the spread of the round goby to new areas within Canada and the United States (Steingraeber, 1999; Wegman, 2006). One suggested tactic is the use of piscicides, such as rotenone. Schreier *et al*. (2008) have suggested that the effectiveness of rotenone and other currently registered piscicides are somewhat limited by the fact that none of these agents tested on round gobies exhibit selectively higher mortality in the round goby compared to native species. Consequently, we are interested in investigating a novel, pheromone-based approach that will allow an efficient and cost-effective method of controlling the spread of the round goby to protect eggs and young of indigenous species.

Figure 7. A photograph of a round goby (*Neogobius melanostomus*, Perciformes: Gobiidae) obtained from Lake Erie at Leamington, Ontario.

1.3.2 Putative Reproductive Pheromones in the Round Goby

Since low light levels prevail in locations that females find the nests occupied by males, reproductive pheromones may be an important part of facilitating reproduction in this species. In previous studies, reproductive females (RFs) were differentiated from non-reproductive females (NRFs) based on ovulation (Belanger *et al.*, 2004; Gammon *et al.*, 2005). Therefore, only pre-ovulatory and ovulated females were considered reproductive and juvenile and post-ovulatory females were considered non-reproductive.

Belanger *et al.* (2004) measured electro-olfactogram (EOG) responses to reproductive male-conditioned water and found strong responses from RFs. The EOG is an electrophysiological method that involves recording graded generator potentials from the olfactory epithelium (Caprio, 1995). A behavioural attraction

assay by Gammon *et al.* (2005) found that delivery of reproductive male (RM)-conditioned water into a tank caused reproductive females to spend more time near the odour source. These results are significant because they suggest that there is likely a pheromone released by reproductive males that attracts RFs.

Several 18-, 19-, and 21-carbon steroidal compounds in particular have been studied as putative reproductive pheromones from endocrine studies of other Gobiid species. These compounds include 5β-androstan-3α-ol-17-one (etiocholanolone, ETIO) and etiocholanolone-glucuronide (ETIO-g), which are synthesized by the testes of males black gobies and attract reproductive females and induced egg deposition (RFs) (Colombo *et al.*, 1977; Colombo *et al.*, 1980). However, there has been no evidence thus far that ETIO-g is synthesized by the testes or seminal vesicles of the round goby (Arbuckle *et al.*, 2005; Jasra *et al.*, 2007).

Murphy *et al.* (2001) performed EOG and gill ventilation studies on a variety of synthetic steroids and prostaglandins in the round goby. In that study, synthetic ETIO, ETIO-g, and ETIO-s were found to elicit both EOG and increased gill ventilation responses in females. Not surprisingly, there is no evidence that the black goby pheromone, ETIO-g, is synthesized by the gonadal tissues of the round goby (Arbuckle *et al.*, 2005; Jasra *et al.*, 2007). Concerning EOG responses by round goby RFs to fractionated reproductive male-conditioned water: fractions containing molecules with chemical characteristics of the black goby pheromones (ETIO or ETIO-g) failed to evoked strong responses, compared to fractions containing alternate steroid-like molecules (Belanger *et al*. 2004). Consequently, Belanger *et al.* (2004) suggested that ETIO or ETIO-g play minor a role in round

goby pheromonal communication, and that the round goby pheromones are other steroidal molecules. Several steroids, including 3α-hydroxy-5β-androstan-11,17-dione (11-oxo-ETIO) were later isolated from the testes and seminal vesicles of RM round gobies (Arbuckle *et al.*, 2005; Jasra *et al.*, 2007). When injected with gonadotropic-releasing hormone (GnRH, stimulates the release of sex-related hormones by stimulating the hypothalamic-gonadal axis) male round gobies significantly increased release rates of 11-oxo-ETIO (conjugated and unconjugated) (Kereliuk *et al.*, 2009). Unconjugated 11-oxo-ETIO also elicits potent EOG responses from females (Laframboise *et al.*, 2008). By virtue of these studies, we expect that 11-oxo-ETIO or a conjugated form may be released as the sex pheromone by male round gobies.

Corkum *et al.* (2008) tested attraction of females to steroid blends of nine conjugated and unconjugated steroids (ETIO, ETIO-g, ETIO-s, 11-oxo-ETIO, 11-oxo-ETIO-g, 11-oxo-ETIO-s, Ad, 11β-OH-Ad and 11-KT). All of these steroids with the exception of ETIO-g are present in the testes of male round gobies. Although reproductive females were not significantly attracted to or repelled by the steroid blends, non-reproductive females were attracted to the unconjugated steroid blend and repelled by the conjugated steroid blend. This suggests that there may be some other steroid responsible for attracting reproductive females.

Dr. Yogesh Katare and Dr. A.P. Scott performed an enzyme-linked immunosorbent assay (ELISA) against 11-oxo-ETIO and conjugates in HPLC-fractionated GnRH-injected male conditioned water (Kereliuk *et al.*, 2009; Katare *et al.*, in preparation). Two fractions, eluting at minute 24 (fraction 24) and minute 30 (fraction 30), were immunoreactive to the anti-11-oxo-ETIO antibody. The

immunoreactive compound in the fraction eluting at minute 30 was identified to be 11-oxo-ETIO by comparing its elution time with that of the standard (synthetic) 11-oxo-ETIO, as well as by determining the mass of the compound through electrospray ionization mass spectrometry. The immunoreactive fraction eluting at minute 24 contains an unknown conjugate or conjugates of 11-oxo-ETIO. When the same fractionation and immunoassay procedure was performed on urine isolated directly from GnRH-injected males, immunoreactivity was detected only in the fraction appearing at minute 24 (containing the conjugated 11-oxo-ETIO). This shows that free 11-oxo-ETIO is not released in the male urine, but by other parts of the body, such as the gills or in the feces. Further, these biochemical studies suggest that there are conjugates of 11-oxo-ETIO released in the reproductive male urine.

This study will expand upon these recent physiological, behavioural, and biochemical progress towards the identification of the reproductive pheromones used by the round goby. Our focus will be on investigating behavioural responses to a blend of synthetic steroids, the urine and methanol extracts of urine from GnRH-injected males, as well as pooled fractions of male-conditioned water that contain 11-oxo-ETIO and its conjugates.

1.4 OBJECTIVES AND SIGNIFICANCE

As part of a project to identify round goby pheromones, we sought to investigate the behavioural responses of reproductive females (RFs) and non-reproductive females (NRFs) to urine from GnRH-injected RMs and to fractionated male-conditioned water. This study also introduced a novel high-throughput fractionation-attraction approach to isolating pheromones. Attraction was measured by combining analysis of the time spent near the odour delivery zone and swimming activity (Belanger *et al.*, 2004; Gammon *et al.*, 2005). Gill ventilation responses were also measured, as female round gobies have been shown to increase gill ventilation rates in response to steroids (Murphy *et al.*, 2001). Particular attention was paid to analyzing the behavioural responses to isolates (prepared by Dr. Y. Katare, University of Windsor) known to contain free 11-oxo-ETIO and the unknown conjugate of 11-oxo-ETIO.

There were four main objectives of this study:

1) **To validate a novel high-throughput technique to study behavioural responses in the round goby.**

Previous behavioural studies in the round goby have used relatively large tanks to test fish one at a time. Our study used a high-throughput fractionation-attraction approach, and we used attraction responses to progress towards the narrowing down of specific HPLC fraction pools of the male conditioned water. In order for this method to be effective, females showed meaningful behavioural responses to delivered odours. The robustness of this assay was tested with an amino acid food odor. In this experiment, we expected that females would not respond to the negative control, and would respond to the food odour.

2) **To test behavioural responses to synthetic steroids.**

Previously, it was found that 5α-reduced steroids did not stimulate gill ventilation in female round gobies (Murphy *et al.*, 2001). As a result, only 5β-reduced steroids and not 5α-reduced steroids were tested in this study. Corkum *et al.* (2008) tested blends of nine synthetic steroids, including ETIO, 11-oxo-ETIO, ETIO-g, ETIO-s, 11-oxo-ETIO-g, 11-oxo-ETIO-s, Ad, 11β-OH-Ad, and 11-KT. With the exception of ETIO-g, all of these steroids are present in the gonadal tissues of male round gobies (Arbuckle *et al.*, 2005; Jasra *et al.*, 2007). Since Ad, 11β-OH-Ad, and 11-KT are also found in other species of fishes (Sorensen & Stacey, 1999; Sorensen *et al.*, 2005b), it is not likely that these are used as reproductive pheromones by round gobies.

In the present study we used small arena high-throughput apparatus for testing a steroid blend that included 11-oxo-ETIO, ETIO-s, ETIO-g, 11-oxo-ETIO-s, and 11-oxo-ETIO-g, as these steroids showed behavioural responses in previous studies. We hypothesized that this blend of synthetic steroids would attract RFs and NRFs.

3) **To determine the attractiveness of urine and methanol extracts of urine from GnRH-injected RMs to females, in comparison to the non-urine portion of male-conditioned water.**

If the reproductive pheromone is released in the urine, the urine should be highly attractive to RFs compared to the non-urine component. If the reproductive pheromone is a steroid molecule, it should partition to the solid phase when the urine is passed through C18 cartridges and should get extracted with methanol from C18 cartridges thus making the methanol extracts attractive to RFs.

4) **To determine the attractiveness of specific pooled fractions of the methanol extracts from male-conditioned water to females.**

HPLC-fractionation was used to separate the compounds present in methanol extracts prepared from GnRH injected reproductive male-conditioned water according to their polarity, which allowed us to test responses to pools of fractions. The fractions were pooled in groups of ten at first, and then attractive pools were further subdivided into groups of five. The fraction pool containing the reproductive pheromone should be highly attractive to RFs.

The use of this high-throughput fractionation-attraction approach is important to progress towards elucidation of the chemical structure of reproductive pheromones in the round goby. Such a process has been used to successfully pinpoint pheromonal compounds in other animals. For example, Linn & Roelofs (1989) were able to isolate different compounds released by female moths using HPLC and test behavioural responses of males to the various components. If a multi-component pheromone is used by the round goby, we may be able to use this fractionation-attraction approach to assess behavioural responses to various portions of exudates from males.

Knowledge of the pheromonal communication system in the round goby not only contributes to the study of Perciform fishes, which are the largest order of extant fishes, but also contributes to the development of pest management practices. Pheromones have been used in the past to successfully control insect pests (reviewed in Corkum & Belanger, 2007). In addition, ongoing developments to a pheromonal control system show promising results for the control of the sea lamprey (Johnson *et al.*, 2009). The behavioural and physiological data presented

in this thesis may aid in the development of similar methods to control the increasing populations of round gobies in the Great Lakes and prevent the spread to other inland waters.

2 MATERIALS & METHODS

2.1 EXPERIMENTAL ANIMALS

2.1.1 Collection & Housing

Round gobies (*Neogobius melanostomus*) (N=144) were collected by angling from Lake Erie at Leamington and Colchester, ON, and from the Detroit River at Windsor, ON during the months of May through October. Collection occurred during the morning and fish were immediately transported to the University of Windsor Animal Quarters in 50 L insulated coolers. Males (n=82) collected were an average length of 11.00 ± 0.3 cm and females (n=62) were an average length of 9.70 ± 0.3 cm. Fish were held in accordance with the University of Windsor Animal Care Guidelines. After overnight acclimation in coolers containing lake water mixed with dechlorinated tap water from the University of Windsor's water supply, fish were transferred either 205 L or 50 L, gravel-lined, aerated tanks held at ca. 18°C. Tanks were either on flow-through or recirculated (filtered: sponge and activated charcoal) dechlorinated water. Fish were held on a constant photoperiod (16 L : 8 D) and provided with ca. 15 cm segments of polyvinyl chloride (PVC) piping for shelter. Fish were fed several times per week with Nutrafin® fish flakes (Tetramin, Inc.).

2.1.2 Reproductive Status

i. Determination of Sex

Male and female round gobies were differentiated based on the appearance of the urogenital papilla. Male round gobies have an elongated,

triangular papilla, whereas female round gobies have a broad, rounded papilla with the appearance of two lobes (Belanger *et al.*, 2004).

ii. Determination of Reproductive Status

Females were used for behavioural experiments. Reproductive females (RFs) were differentiated from non-reproductive females (NRFs) based on the presence of eggs, which cause the abdomen to become visibly swollen. The overnight acclimation period after collection, in which fish were not fed, ensured that the swollen abdomen was a result of the eggs and not food consumption, which would be excreted.

Males were used for collection of urine, and collection of male-conditioned water. Reproductive males (RMs) were subjectively differentiated from non-reproductive males (NRMs) prior to euthanization by secondary sexual characteristics, which include dark body, swollen cheeks, swollen urogenital papilla, larger fins, and the presence of a thick slime coat. After euthanization, reproductive status of the males was confirmed on the basis of gonadal somatic index (GSI), which is defined as follows:

GONAD WEIGHT / TOTAL WEIGHT x 100%

Gonad weight includes the testes, seminal vesicles, and mesorchial gland. Males with GSI of over 1.3 were considered reproductive (Belanger *et al.*, 2004; Gammon *et al.*, 2005). Only RMs were used for this study, as Gammon *et al.* (2005) have shown that females do not respond to non-reproductive male-conditioned water.

2.2 PREPARATION OF TEST ODOURS

For a summary of the GnRH injection, odour collection, and behavioural assay process, see **Figure 8**. For a summary of all the odours prepared and brief descriptions of the contents of each, see **Table 1**.

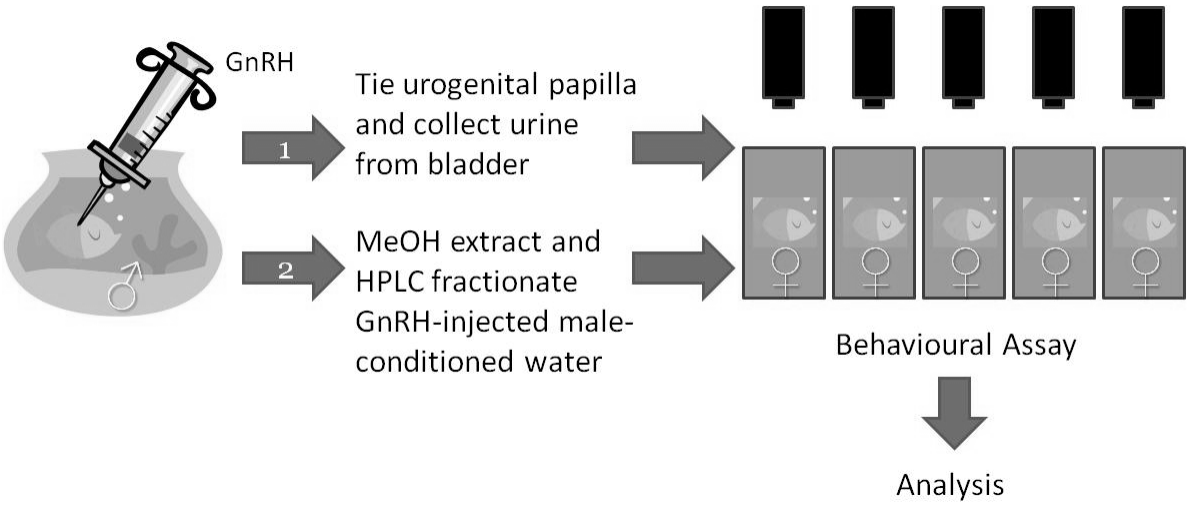

Figure 8. A summary of the test sample preparation and the behavioural assay process. After males were injected with GnRH to prime the hypothalamic-gonadal axis, urine or conditioned water was collected and delivered to females in the assay tank set-up.

Table 1. An overview of the all the test solutions delivered to female round gobies in the behavioural arena.

Test Solution	Contents and Purpose
Vehicle Blank	Food Odour trials: dechlorinated water Fraction trials: dechlorinated water run through methanol extraction Control for odour contamination, mechanical effects
Food Odour	10^{-5} M L-alanine in dechlorinated water - positive control – should elicit attraction in hungry females
Synthetic Steroid Blend	Synthetic 11-oxo-ETIO, 11-oxo-ETIO-s, 11-oxo-ETIO-g, ETIO-s, and ETIO-g ethanol stocks diluted to 10^{-6} M in dechlorinated water
Urine from GnRH-injected RMs	This urine contains conjugates of 11-oxo-ETIO, but not free 11-oxo-ETIO.
Non-urine conditioned water	Mixture of substances (including free 11-oxo-ETIO) released from other parts of the body than by the urogenital papilla
Methanol extracts of urine	Solid phase from extraction of urine (non-polar compounds)
Aqueous run-off from urine	Aqueous phase from extraction of urine (polar compounds)
Fraction Pools 1-11, 11-21	Relatively polar molecules released into the methanol extracted and HPLC fractionated whole male-conditioned water
Fraction Pool 21-31	Includes 11-oxo-ETIO and its conjugates
Fraction Pool 21-26	Includes unknown conjugate(s) of 11-oxo-ETIO
Fraction Pool 26-31	Includes free 11-oxo-ETIO
Fraction Pool 31-40	Non-polar molecules released into the whole male-conditioned water
Aqueous run-off from fractions	Aqueous phase from the extraction of the whole male-conditioned water (polar compounds)

2.2.1 Conditioned Water from Reproductive Males

The production of sex-related hormones by RMs was enhanced by stimulating the hypophyso-gonadal axis with *s*-GnRH. After acclimation to laboratory conditions, males that appeared dark in colour with swollen urogenital papillae (MacInnis & Corkum, 2000) were isolated in 1 L of dechlorinated, aerated water. These fish were allowed to acclimate to these tanks for four hours before given a 0.5% body weight *s*-GnRH (Syndell Labs, Vancouver, BC) injection of 0.7% GnRH dissolved in 0.9% NaCl with distilled water as a vehicle. After injection, either urine (see section 2.2.2) or conditioned waters (see section 2.2.3) were collected.

2.2.2 Urine Collection

The urine preparations that were tested for behavioural responses represented the amount of urine produced by a single RM injected with GnRH, and held in 1 liter of water over a 4 hour period. This urine was collected from the bladder following ligation of the urogenital papilla; and urine from 3 to 5 RMs was pooled for behavioural assay. Initially, males were acclimated in 1 L containers for two hours after GnRH injection, then lightly anesthetized with 0.6% 2-phenoxy ethanol (Sigma-Aldrich, Oakville, ON, CAS #122-99-6) and the urogenital papilla was tied shut with fishing line and the water in the container was changed. The fish was left in the container for an additional 4 hours to allow urine to collect in the closed bladder, then the fish was sacrificed and urine was collected directly from the distended abdomen using a syringe (1 mL, 26G 3/8 tip). Urine collected from reproductive males was pooled and diluted with dechlorinated water to simulate

natural dispersion conditions calculated based on the volume of water in which males were held prior to euthanization (by Dr. Y. Katare). Final urine dilutions ranged from $1:10^4$ to $1:10^5$. Additional urine was solid-phase extracted to separate the solid phase from the aqueous run-off (C18 Sep Pak, Waters, Milford, MA). Non-polar compounds such as steroids should separate out to the solid phase rather than the aqueous phase. The methanol extracts from this urine were dried using CentriVap vacuum concentrator system (Model # 7984010, Labconco, Kansas City, MO), reconstituted and diluted with same proportion of dechlorinated water as urine was diluted. The conditioned water left in the container after urine collection was also collected for odour delivery on the assumption that ligation prevented any urine from moving into the water.

After injection and collection of urine and conditioned water, males were euthanized with an overdose of MS-222 (5% tricaine methane sulfonate, Finquel®, Argent Chemical Lab) and GSI measurements were taken. Urine and conditioned water from males that had a GSI of lower than 1.3 were not included for behavioural testing, as Gammon *et al.* (2005) have found that females were not attracted to conditioned water from non-reproductive males (NRMs).

2.2.3 Collection of HPLC Fractions of Male-Conditioned Water

For conditioned water collection for fraction trials, GnRH-injected males were held in the 1 L of dechlorinated water for 16 hours (no ligation was performed, and males were allowed to urinate into the water). After this period, the water was collected and solid phase extracted (C18 Sep Pak, Waters, Milford, MA). The methanol extracts were then run through high performance liquid

chromatography (HPLC) on an ODS-M80 (4 µm) column (Waters, Milford, MA) (Dept. of Chemistry & Biochemistry) using a linear gradient from 10% acetonitrile (ACN) (0.1% trifluoroacetic acid, TFA) : 90% Water (0.1% TFA) to 90% ACN (0.1% TFA) : 10% Water (0.1% TFA) over 40 minutes (performed by Dr. Yogesh Katare). The flow rate was 0.5 mL per minute. The HPLC process separates molecules in the conditioned water based on polarity, with polar molecules eluting first and non-polar molecules eluting later (**Figure 9**). Following HPLC fractionation, the fractions were pooled into the following test groups corresponding to the minutes at which they eluted from the column: fractions 1-11, 11-21, 21-26 (contains the unknown conjugate of 11-oxo-ETIO), 26-31 (contains free 11-oxo-ETIO), and 31-40. The pooled fractions were evaporated in the CentriVap® and reconstituted with dechlorinated water to an equivalent concentration to 10^{-8} M 11-oxo-ETIO (i.e., the concentration of 11-oxo-ETIO in the fractions was 10^{-8} M, and other unknown molecules were at varying concentrations based on the dilution used to make 10^{-8} M 11-oxo-ETIO based on an ELISA performed by Dr. Y. Katare). A vehicle blank comprising of 50:50 (v/v) mixture of acetonitrile and water containing 0.1% TFA evaporated and reconstituted in dechlorinated water was also prepared. This procedural control odour was delivered as to the females to test for responses to contamination arising from the purification process as well as mechanical effects from switching the odour. We expected that females should not respond to delivery of this vehicle blank.

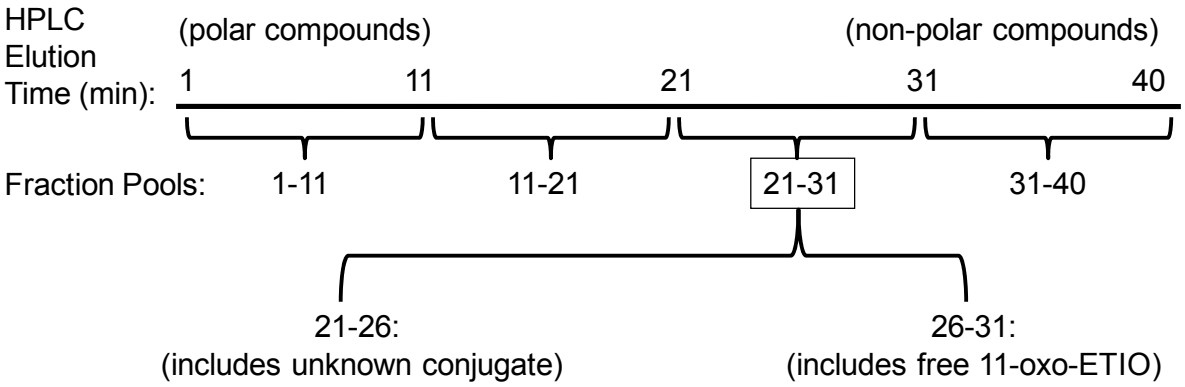

Figure 9. An overview of the HPLC process showing the relationship between elution time (in minutes) and polarity of eluted compounds. The fractions were pooled in groups of ten for the first round of behavioural testing, and for subsequent tests, fractions 21-31 were broken down to 21-26, which contains the unknown conjugate of 11-oxo-ETIO, and 26-31, which contains free 11-oxo-ETIO

2.2.4 Preparation of Synthetic Odours

The amino acid, L-alanine (Sigma Aldrich, CAS #56-41-7) was prepared as a food odourant as a positive control. Food odourants should be attractive to females (Papatryphon & Soares, 2000). The L-alanine was diluted to 10^{-5} M in dechlorinated water (background water). It is important to note that, since L-alanine is a food odour, there may be taste-related behavioural responses to L-alanine. In several species, it has been found that amino acids are detected not only in the olfactory systems, but by taste buds as well (reviewed in Hara, 1994; 2006b). A similar trend is expected here.

A blend of synthetic steroids (all obtained from Steraloids, Inc., Newport, RI) consisting of 11-oxo-ETIO (CAS #739-27-5), 3-sulphated 11-oxo-ETIO (Steraloids Catalogue #A3500-000), 3-glucuronated 11-oxo-ETIO (CAS #17181-16-7), ETIO-sulphate (CAS #2681-45-0), and ETIO-glucuronide (CAS #3602-09-3) was also delivered. This differs from the blends used by Corkum *et al.* (2008), as we excluded those free steroids which were not 5β reduced and also a 5β reduced steroid ETIO (which is only a minor product of male round goby testes according to Arbuckle *et. al.*, 2005) from the blend. Note that our steroid blend did not contain the unknown conjugate of 11-oxo-ETIO described in chapter 1. The steroids were prepared from 1 mg/mL ethanol stocks, and diluted to concentrations of 10^{-6} M in dechlorinated water, which diluted to a theoretical final concentration of 10^{-8} M in the assay tanks.

2.3 BEHAVIOURAL ASSAY AND ANALYSIS

2.3.1 Behavioural Tanks

Female round gobies were isolated and held in individual behaviour tanks on aerated and dechlorinated flow-through water held at 18 ± 2°C with one fish per tank (**Figure 10**). Each behaviour tank held 5 L of water and had dimensions of 30 cm by 15 cm. The fish were allowed to acclimate to the tanks for 24 hours after transfer from holding tanks prior to behavioural testing. The behavioural tanks were constructed with an inflow and outflow so that there was a constant flow-through of dechlorinated water using a peristaltic pump (Cole-Parmer model #7519-06, Vernon Hills, IL) at a rate of 5 mL/min (tubing: L/S 14 Pt-cured silicone, Masterflex, Vernon Hills, IL).

Olfactory Deprivation

In order to confirm that responses we saw were olfactory responses, we deprived six female round gobies (3 RF and 3 NRF) of their olfactory abilities as in (Belanger *et al.*, 2006). We applied Reprosil® dental caulking (Vinyl Polysiloxane, Dentsply Caulk, York, PA) to the nares using a 26G 3/8-tipped syringe, which Belanger *et al.* (2006) showed effectively blocks odours from entering the nares. If the responses are only olfactory responses, the olfactory deprived females should not respond to any of the odours delivered except the food odour, which may act as a tastant.

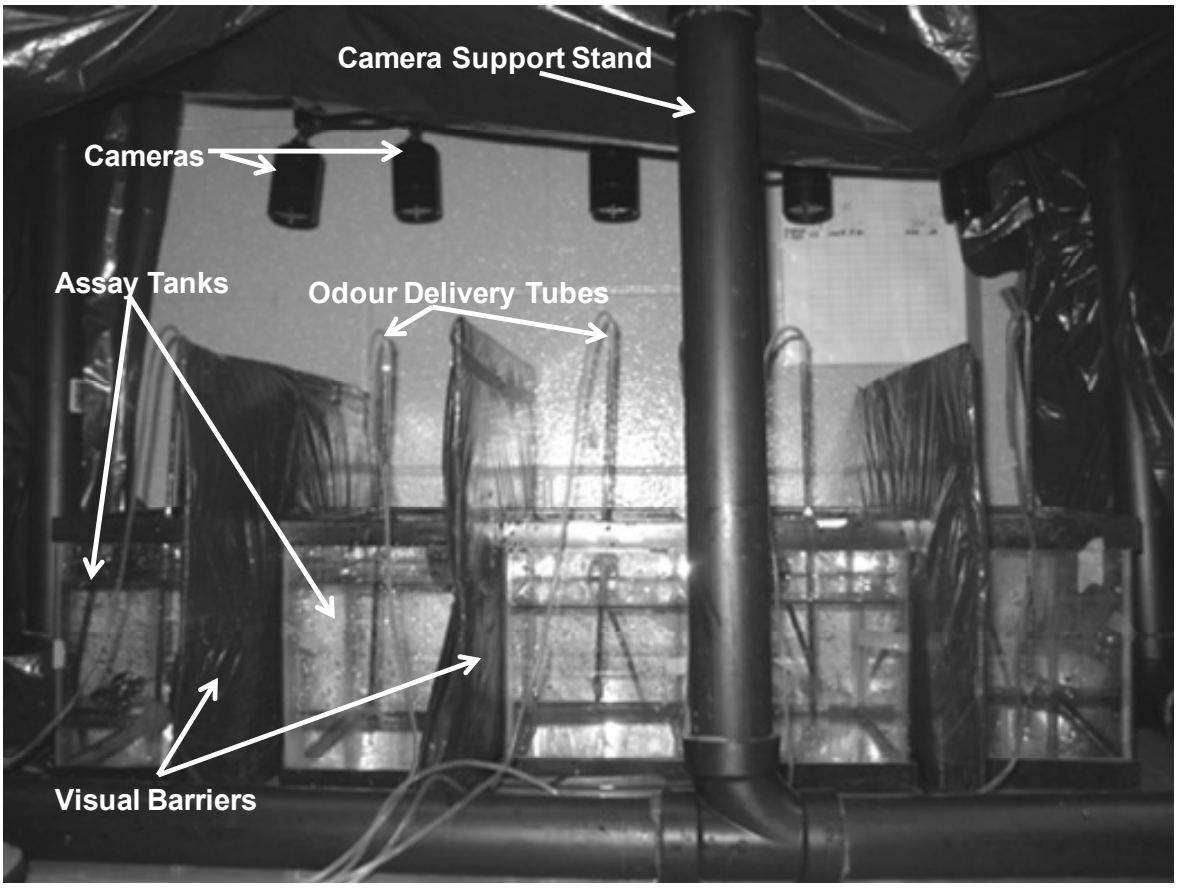

Figure 10. Five-tank behavioural assay set-up with inflow tubes and drains. Barriers were placed between the tanks to prevent fish from seeing each other. Each tank is below an infrared digital video camera, which send video to a computer (not shown).

2.3.2 Behavioural Tank Calibration

In order to determine approximate odour concentrations in the assay tanks during odour delivery, red food dye (Club House®, London, ON) was delivered and samples of tank water were taken from various points along the bottom of the tank with a pipette (Pipetman® P1000®, Gilson, Middleton, WI) (since gobies are benthic animals) at 1 min after odour delivery, 5 min after odour delivery, and 11 min after odour delivery following the application of known dilutions of food colouring. A standard curve was prepared with a spectrophotometer (Agilent 8453, Santa Clara, CA) and compared to the tank samples to estimate approximate odour dilutions around the tank.

In order for our assay to be effective, there must be a concentration gradient after odour delivery. The fish will swim towards the high concentration zone and stay in if the odour is attractive (Gammon *et al.*, 2005). We conducted dye trials to determine estimated odour diffusion patterns in the assay tanks. After delivery of the dye, there is a zone of concentrated dye around the odour delivery tube within the 25% of the tank closest to the tube. Based on the delivery of 50 mL of odour into a 5 L tank, the theoretical final tank dilution after complete dispersion (without drainage) would be a factor of 0.01.

The dye calibration trials repeated 10 times determined that the estimated odour dilution in the inflow zone would range from a factor of 0.003 ± 0.001 to 0.019 ± 0.012 after 1 min, from 0.006 ± 0.002 to 0.039 ± 0.016 after 5 min, and from 0.009 ± 0.003 to 0.035 ± 0.008 after 11 min (**Figure 11**). This validated the use of the inflow zone.

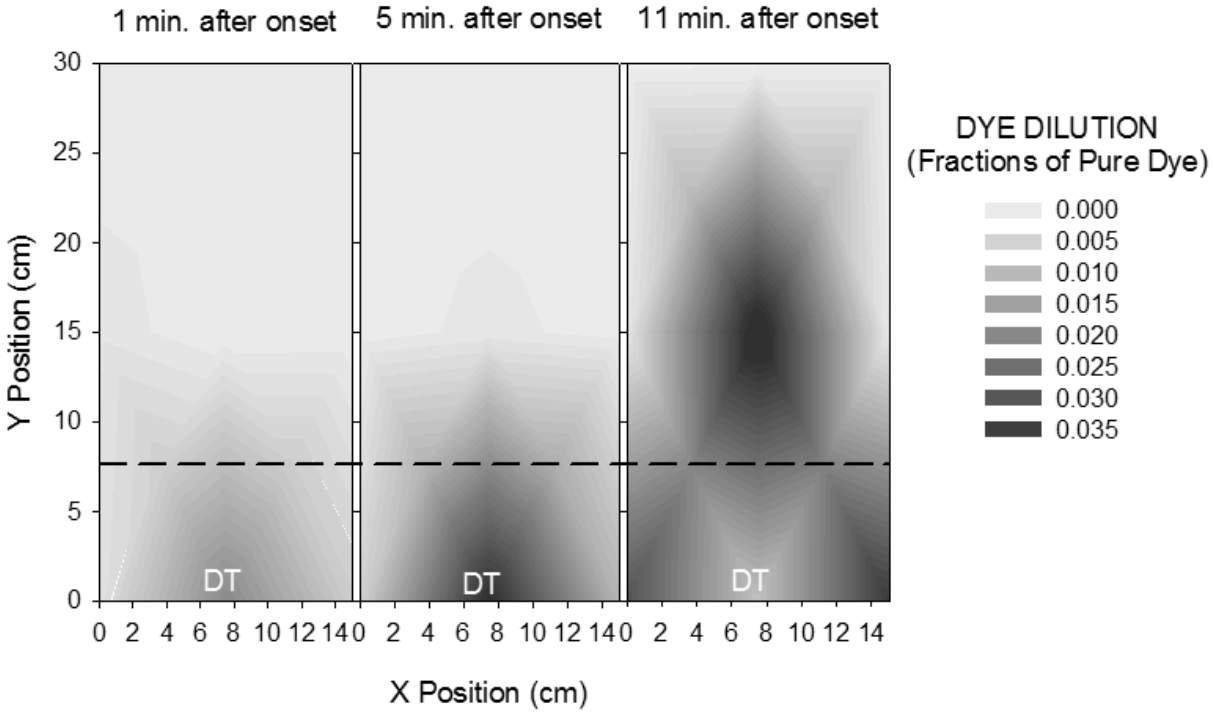

Figure 11. Contour plots showing average (over ten trials) dye dilutions (expressed as fractions of the pure dye delivered) at given points throughout the assay tanks at 1 minute, 5 minutes, and 11 minutes after the onset of dye delivery. Dye was delivered for 10 minutes. The dashed line denotes the boundary of the inflow zone, and DT denotes the location of the dye delivery tube.

2.3.3 Behavioural Testing of Odourants

Behavioural experiments were conducted on reproductive and non-reproductive females to determine if there were behavioural responses to the urine from GnRH-injected males and to the component fractions of the male-scented water. Odours were delivered by switching the peristaltic pump intake to a flask containing the test solution for a 10 minute delivery period, followed by returning the intake to background water to allow the odour to wash out for at least 30 minutes. The order of the odourants delivered on a given experimental day was randomized each time. Behaviour was recorded digitally using infrared cameras (AX-808C-SH, Matco, Inc., St. Laurent, QC) and analyzed.

2.3.4 Data Analysis & Statistical Methods

Swimming and gill ventilation responses were analyzed during this study. The video was digitized and run through the tracking software Ethovision® XT (Version 5, Noldus, Leesburg, VA), which tracks movement within the behavioural arena (**Figure 12**). To quantify behaviour, we measured time spent in the inflow zone (the 25% of the tank closest to the odour delivery tube; modified from Gammon *et al.* (2005)), swimming activity, distance travelled. Gill ventilation responses were also monitored. Since gobies are sessile animals, only fish that were actively swimming were used for experimentation. Thus, fish that spent more than 60% of their time in the high concentration zone in the five minutes prior to delivery were excluded from the swimming trials but were still included in gill ventilation trials. All statistical analyses were performed using SigmaStat® 3.5

(Systat, San Jose, CA). For all statistical tests, significant difference was noted for $\alpha<0.05$.

i. Time Spent in the Inflow Zone

As previously stated by Gammon *et al.* (2005), attraction was said to occur if there was an increase in the time spent in the inflow zone five minutes after odour delivery in comparison to the five minutes before odour delivery. We set Ethovision to sample the fish's location 30 times per second. The fish's location was read out as positional data within the calibrated arena (automatic visual calibration based on the width of the tank being 15 cm). Each sample was scored as either a positive (fish is in the inflow zone) or a negative (fish is not in the inflow zone). The percent positive responses in each minute were taken as the percent time in each minute spent in the inflow zone. Change in time spent in the inflow zone was calculated by subtracting the average percent time spent in the zone over five minutes after the onset of odour delivery from the average percent time spent in the zone over five minutes before delivery. Student's t-tests were used to find statistical differences between changes in time spent in zone between RFs and NRFs within each odour treatment. One-way analysis of variance (ANOVA) and Holm-Sidak post-hoc tests were to were used to compare average time spent in zone among odour treatments and to compare treatments to the vehicle blank (described in 2.2.3), which was the negative control. Percent data was arcsine transformed in order to satisfy the requirements of the parametric statistics used.

ii. Swimming Activity and Distance Travelled

Swimming activity was measured using a spike analysis in GraphPad Prism® 5 (GraphPad Software, La Jolla, CA). Average swimming speeds over three second intervals were calculated from the Ethovision XT® position data. Based on visual observations of the swimming videos, a single movement (spike) was said to occur if the fish moved more than 1 cm. The total number of movements in the five minutes after odour delivery was subtracted from the total number of movements in the five minutes before odour delivery to yield the change in total activity. Average distance travelled was measured by plotting average swimming speeds versus time in GraphPad Prism® and calculating the area under the curve. Change in average distance travelled was calculated by subtracting the average distance travelled over the five minutes after odour delivery from the average distance travelled over the five minutes prior to odour delivery. Mann-Whitney non-parametric analysis was used to find statistical differences between changes in distance travelled between RFs and NRFs within each odour treatment. Kruskal-Wallis non-parametric one-way analysis of variance with Dunn's difference of ranks was used to compare the average change in distance travelled among odour treatments.

iii. Gill Ventilation

Average gill ventilation rates were monitored manually from the digital recordings of the fish five minutes before and five minutes after odour delivery to determine physiological responses to odours (Murphy *et al.*, 2001; Belanger *et al.*, 2006; Belanger *et al.*, 2007). The change in gill ventilation rate was calculated by

subtracting the gill ventilation rate (ventilations per minute) over the five minutes after odour delivery from the gill ventilation rate over the five minutes before odour delivery. Mann-Whitney non-parametric analysis was used to determine if there was a statistical difference in the changes in gill ventilation rates between RFs and NRFs. Kruskal-Wallis non-parametric one-way analysis of variance with Dunn's difference of ranks was used to compare changes among odour treatments.

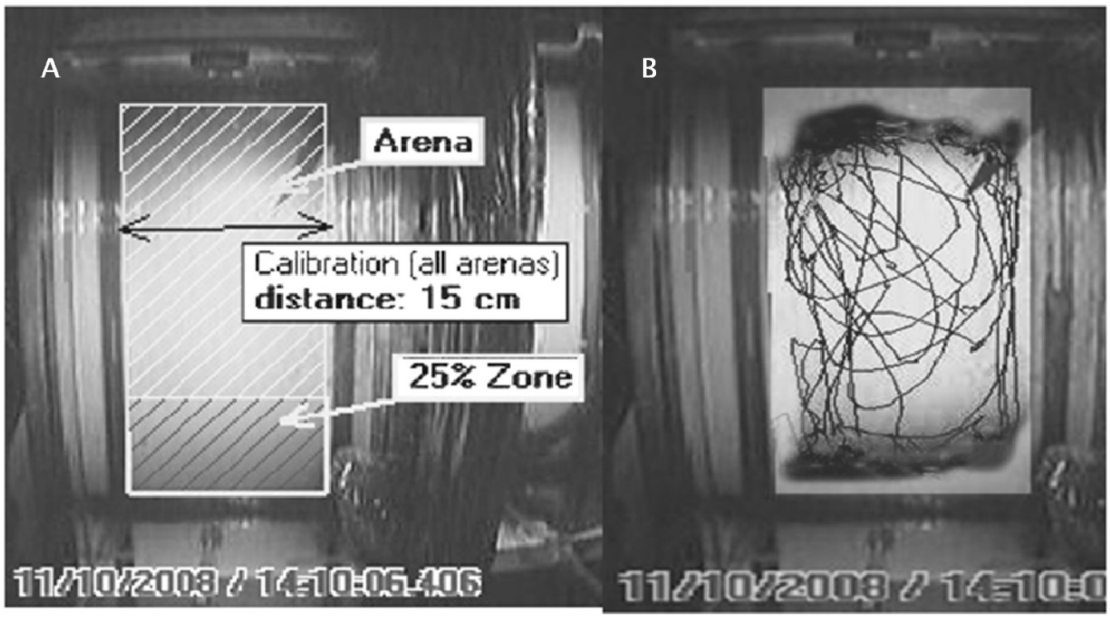

Figure 12. Digital analysis of the behavioural assay tanks with Ethovision XT showing the Arena, inflow zone, and calibration (the width of the tank is set to 15 cm) (**A**), and a sample activity trace of one fish over six hours (**B**).

3 RESULTS

3.1 FEMALE RESPONSES TO L-ALANINE (FOOD ODOUR)

3.1.1 Time Spent in the Inflow Zone

The inflow zone comprised 25% of the tank area closest to the odour delivery tube. When background water was flowing into the assay tanks during the pre-odour delivery periods, females (n=17) spent an average of 29.91 ± 6.42% of the time in the inflow zone. Thus, there was no preference for the inflow.

i. Vehicle Blank – Negative Control

The vehicle blank for amino acids (dechlorinated water) did not elicit a significant change from the baseline when delivered (t_{28}= 1.852, P=0.075) (**Figure 13**). We delivered this vehicle blank to ensure that no contamination had arisen from the odour preparation and that there were no mechanical effects from switching the peristaltic pump. Since there was no preference for the vehicle blank, we can conclude that there were no responses from contamination or mechanical effects in subsequent tests.

ii. Food Odour

Food odours should be attractive to unfed females. When 10^{-5} M L-alanine was added to the tank inflow, females (n=26) increased the average percent time (± SEM) spent in zone of with a peak occurring within the first five minutes after odour delivery, returning to baseline by the time odour delivery is done (**Figure 13**). There was a significant increase in the change in percent time spent in the inflow zone when L-alanine is delivered (19.64 ± 6.32%) compared to the vehicle blank (-14.06 ± 7.51%) (t_{17}=4.939, P<0.001) (**Figure 14**). There was

not a significant difference in the response to 10^{-5} M L-alanine between reproductive females (RFs) and non-reproductive females (NRFs) (t_{23}=0.869, P=0.394). The attraction responses to 10^{-5} M L-alanine validates the high-throughput assay system, as these fish were not fed for at least 24 hours and therefore should show attraction to a food odour. We expected to see similar attraction responses to pheromonal compounds.

In order to ensure the responses we obtained to various odours were actually olfactory-based responses, without the contribution of the other chemical senses, we deprived some females (n=6) of their olfactory sense by plugging both nostrils before running L-alanine, and the vehicle blank. We hypothesized that if the responses we saw were solely olfactory-based, there should not be any responses to these odourants in the olfactory deprived females. Neither L-alanine nor the vehicle blank elicited a significant change in percent time spent in zone from baseline ($F_{3,20}$=2.560, P=0.084) when the nostrils were occluded (**Figure 14**).

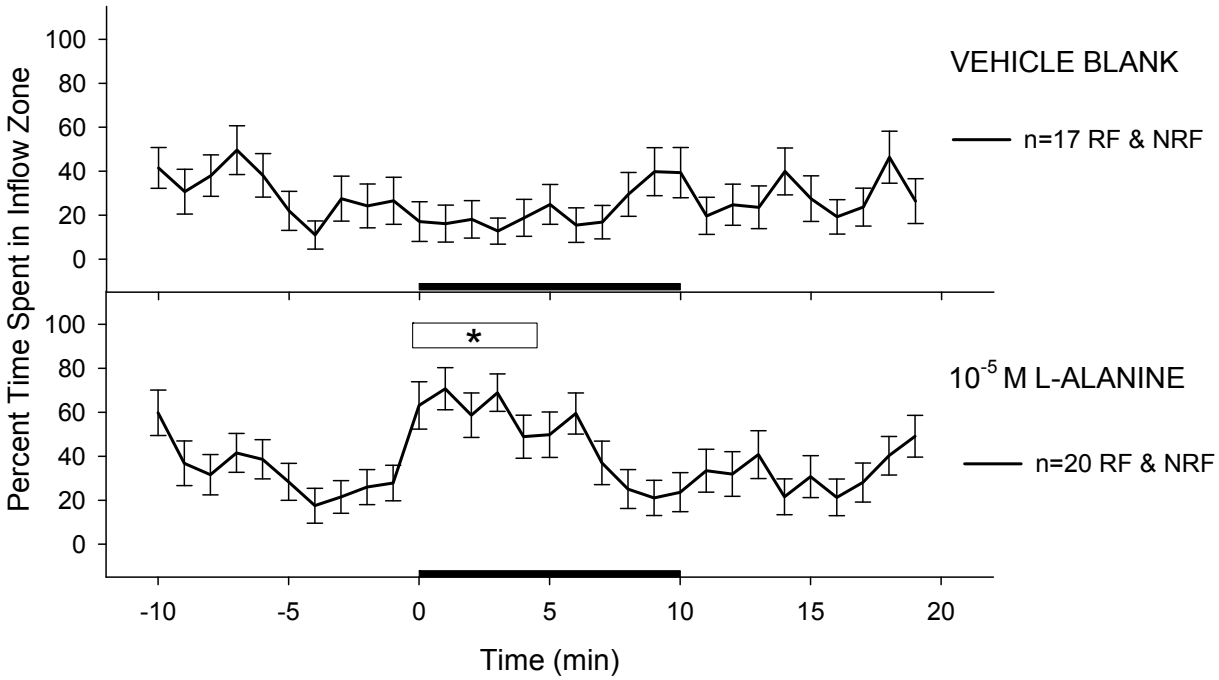

Figure 13. Average percent time (± SEM) spent in the inflow zone after delivery of vehicle blank (negative control) and 10^{-5} M L-alanine. There was not a significant change between the average time spent in the zone in the ten minutes after odour delivery for vehicle blank (P=0.075), as opposed to L-alanine, which exhibits a peak within the first five minutes after odour delivery. Black bars on the x-axis denote odour delivery period. The x-axis is time in minutes, with negative values referring to the pre-odour delivery period. The boxed asterisk denotes significant difference over five minutes from baseline (P<0.05).

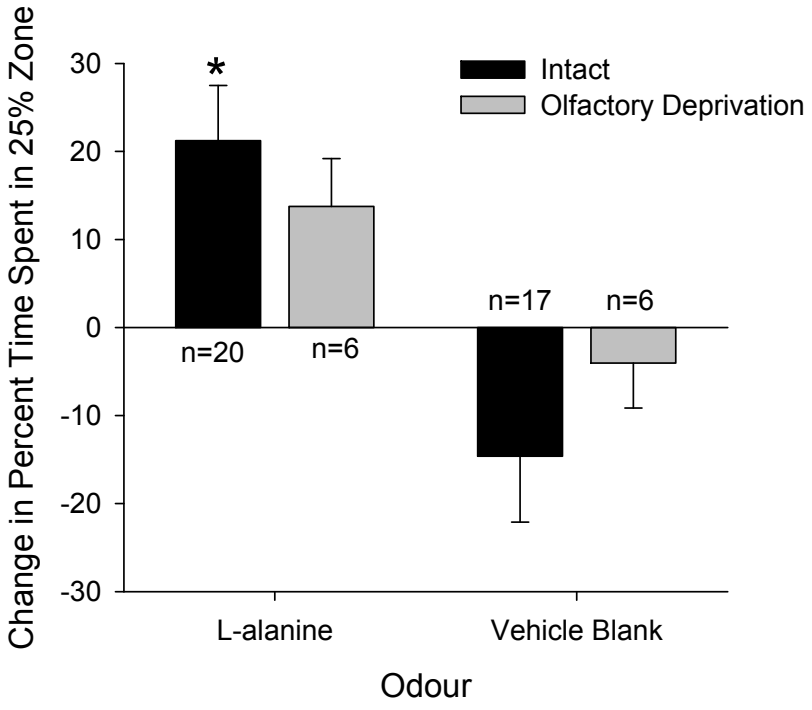

Figure 14. Average change in the percent time spent in the inflow zone (± SEM) after odour delivery for 10^{-5} M L-alanine and vehicle blank. There is a significant increase in the time spent in the inflow zone when females are presented with L-alanine compared to vehicle blank during normal odour delivery (denoted by asterisk, $P<0.001$), but not during olfactory deprivation ($P=0.084$).

3.1.2 Swimming Activity & Distance Travelled

When only background water was flowing into the assay tanks during the five minutes prior to odour delivery period, females (n=17) moved 22 times, and travelled an average distance of 13.23 ± 1.32 cm. This was used as a baseline activity measure for the swimming activity experiments.

i. Vehicle Blank – Negative Control

There was an increase by approximately two movements following delivery of the vehicle blank (dechlorinated water). The vehicle blank did not elicit a significant change in distance travelled from the baseline when delivered (U=172, P=0.349) (**Figure 15**). This suggests that there was no significant change in swimming activity after delivery of the vehicle blank, which controlled for contamination and mechanical effects of switching the odour.

ii. Food Odour

When 10^{-5} M L-alanine was introduced in the inflow of the tank, females (n=26) increased the average movements by 9. There was a significant decrease in average distance travelled when L-alanine is delivered (-22.76 ± 9.00 cm) compared to the vehicle blank (3.78 ± 3.01 cm) (H=29.958, P<0.05) (**Figure 15**). There was no significant difference in the change in distance travelled between reproductive females (RFs, n=12) and non-reproductive females (NRFs, n=13) (U=37.000, P=0.883). The increase in movements and decrease in distance travelled suggests that there were more frequent movements in a smaller area of the tank after delivery of this food odour. Similar swimming activity was expected in response to pheromonal compounds.

As expected, neither L-alanine nor the vehicle blank elicited any significant change in swimming activity or distance travelled ($F_{3,20}$=2.155, P=0.125) when the nostrils were plugged (**Figure 15**), demonstrating the responses to L-alanine were olfactory-based responses.

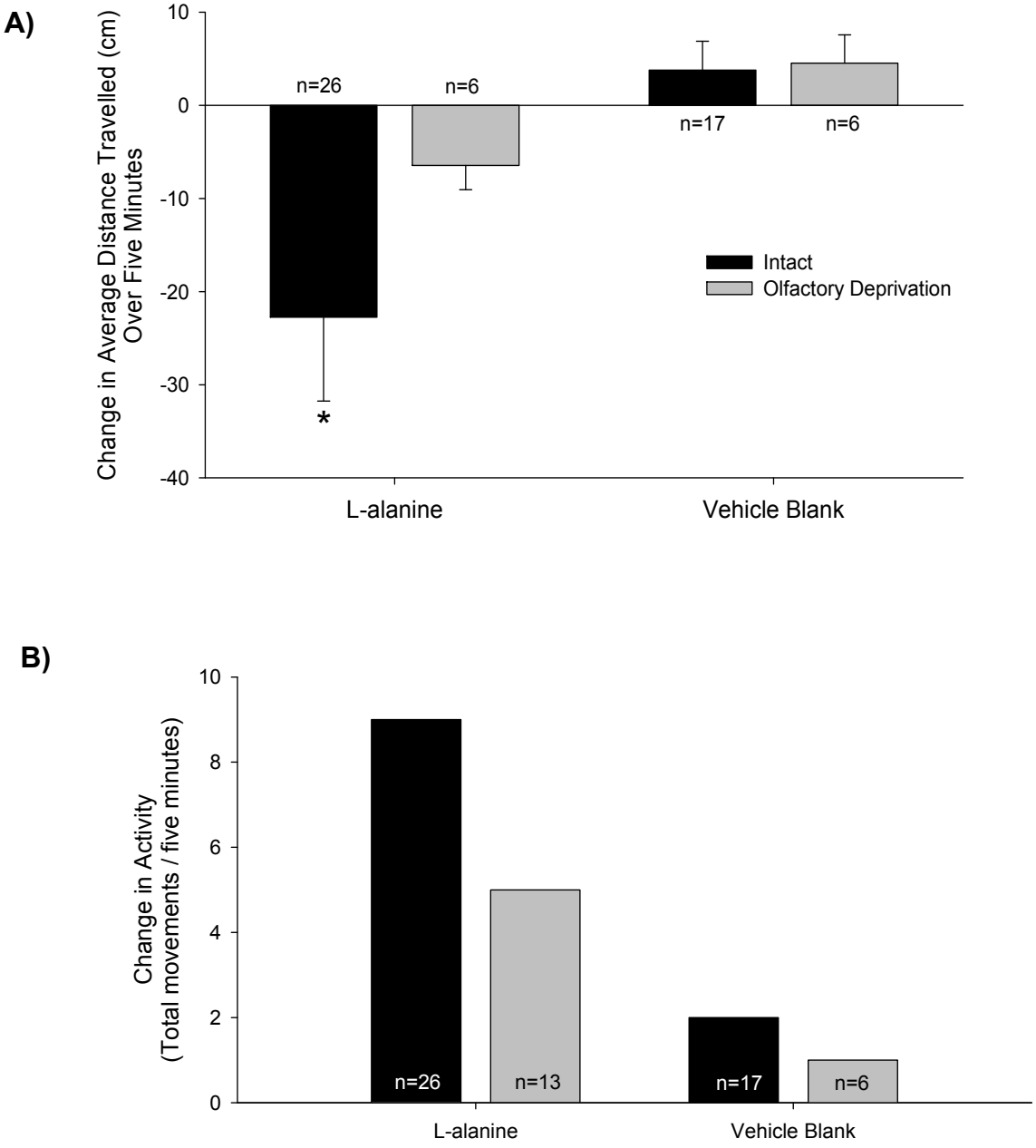

Figure 15. Average change in distance travelled (cm ± SEM) (**A**) and total number of movements (**B**) over the five minutes after odour delivery compared to the five minutes before odour delivery for 10^{-5} M L-alanine (food odour) and vehicle blank (negative control). There was a significant difference in the distance travelled (denoted by asterisk, $P<0.05$) between L-alanine and the vehicle blank for the normal odour delivery only.

3.1.3 Gill Ventilation Responses

As individual fish tended to vary on baseline gill ventilation rates, the change in gill ventilation rate for each individual fish from baseline was measured after odour delivery. The average baseline gill ventilation rate before odour delivery was 44.13 ± 2.01 ventilations per minute (vent/min) (n=30). This value was used as a baseline gill ventilation rate for gill ventilation responses.

i. Vehicle Blank – Negative Control

The vehicle blank of dechlorinated water did not elicit a significant change from the baseline when delivered (0.60 ± 0.29 vent/min) (n=30, U=619.5, P=0.338) (**Figure 16**). This suggests that neither any residual contaminants from the odour purification process nor the switching of the pump for odour delivery affected gill ventilation rate.

ii. Food Odour

When presented with 10^{-5} M L-alanine, all females (n=29) increased the average gill ventilation by 5.45 ± 1.12 vent/min. There was no significant difference between RFs (n=12) and NRFs (n=17) (U=95.0, P=0.771), but there was a significant increase compared to the vehicle blank (U=127.5, P<0.001) (**Figure 16**). This suggests that these fish respond behaviourally to food odours and that this response extends to an increase in gill ventilation.

Neither L-alanine nor the vehicle blank elicited any significant change in gill ventilation rate ($F_{3,20}$=0.962, P=0.430) when the nostrils were plugged (**Figure 16**), suggesting responses to L-alanine are olfactory-based responses.

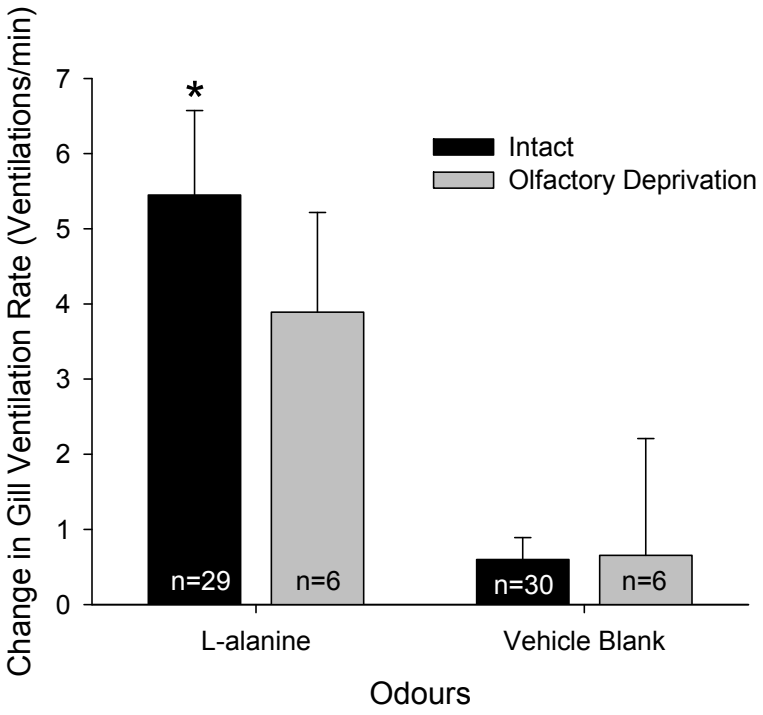

Figure 16. Average change (five minutes after odour delivery minus five minutes before delivery) in gill ventilation rate (ventilations/minute ± SEM) of 10^{-5} M L-alanine (food odour) and vehicle blank (negative control). There was a significant change from baseline for 10^{-5} M L-alanine during the normal odour delivery trials only (denoted by asterisk, $P<0.05$).

3.2 FEMALE RESPONSES TO A BLEND OF CONJUGATED AND UNCONJUGATED SYNTHETIC STEROIDS

This test is based on our overall hypothesis that derivatives of sex steroid(s) are used as pheromones in the round goby. A blend of synthetic five steroids (11-oxo-ETIO, 11-oxo-ETIO-s, 11-oxo-ETIO-g, ETIO-s, and ETIO-g) at a delivery concentration of 10^{-6} M was also tested on females. We performed this test to determine if females are attracted to a blend of synthetic steroids that have previously been linked to increases in gill ventilation. All of these steroids, with the exception of ETIO-g, are released by male round gobies.

3.2.1 Time Spent in the Inflow Zone

When the synthetic steroid blend was delivered via the inflow, both RFs (n=8) and NRFs (n=8) spent more time in the inflow zone within five minutes after odour delivery (**Figure 17**). The females increased their time spent in the zone by 25.21 ± 5.78%, which was significantly different from the vehicle blank ($F_{10,130}$=8.735, P<0.001) (**Figure 18**). There was no significant difference between the responses of the RFs (25.72 ± 8.51%) and NRFs (24.55 ± 9.83%) to the steroid blend ($F_{7,109}$=4.456, P=0.929). This demonstrates that all females showed a preference to the delivered blend of synthetic steroids.

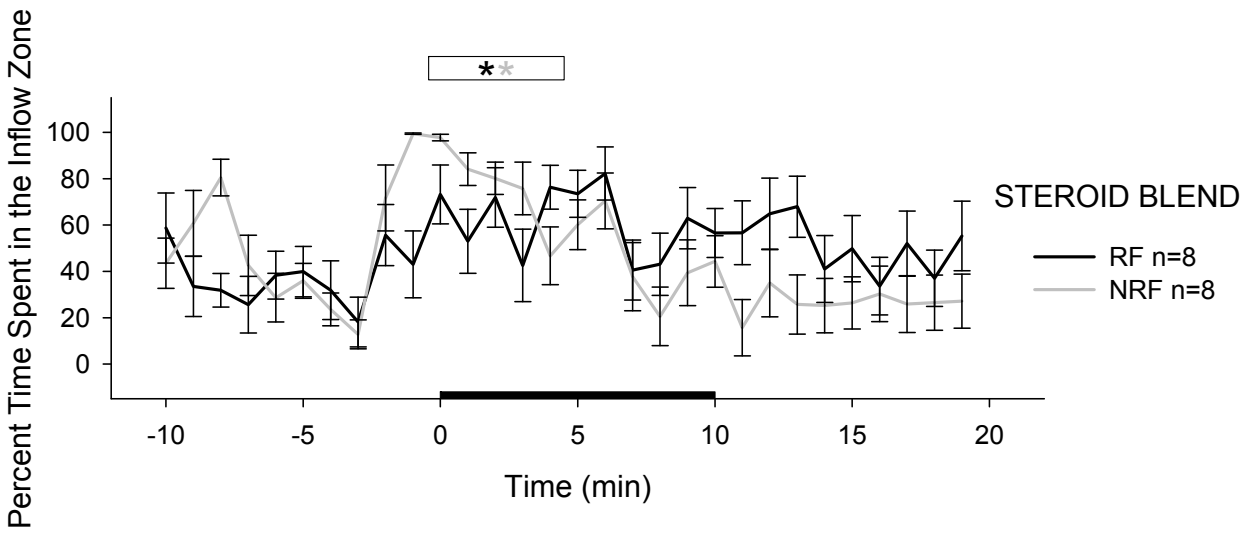

Figure 17. Average percent time (± SEM) spent in the inflow zone after delivery of the synthetic steroid blend containing 10^{-6} M 11-oxo-ETIO, 11-oxo-ETIO-s, 11-oxo-ETIO-g, ETIO-s, and ETIO-g. There was a peak within the first five minutes after odour delivery (black bar) for both RFs and NRFs, followed by recovery to baseline. The x-axis is time in minutes, with negative values referring to the pre-odour delivery period. The boxed asterisk denotes significant difference over five minutes from baseline for each odour ($P<0.05$).

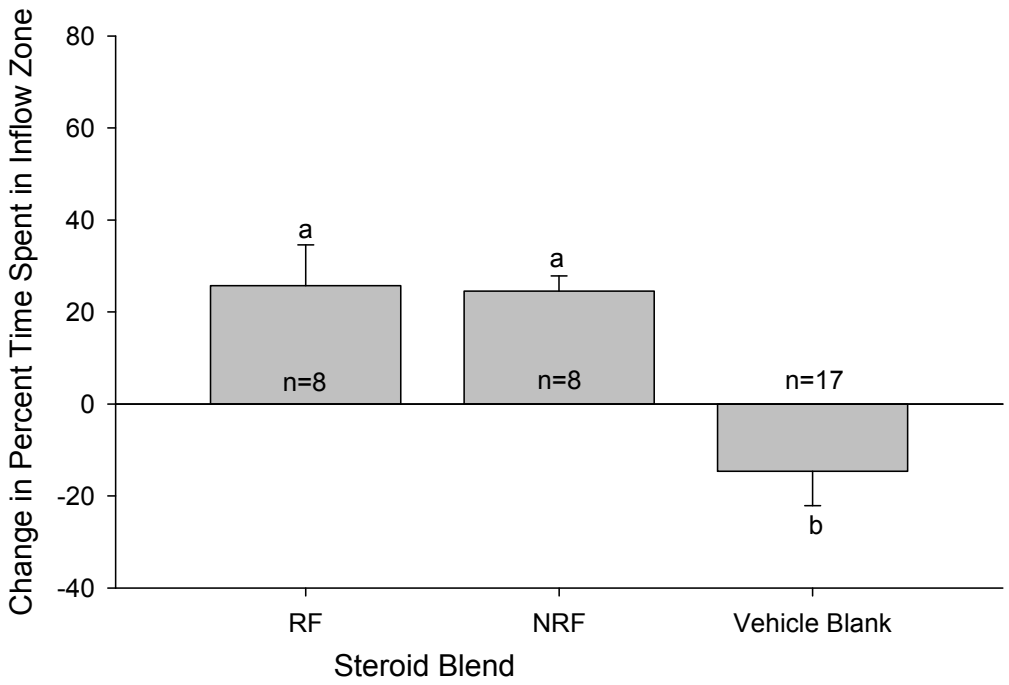

Figure 18. Both the RFs and NRFs significantly increased the average perecent time (± SEM) spent in the inflow zone after delivery of the synthetic steroid blend containing 11-oxo-ETIO, 11-oxo-ETIO-g, 11-oxo-ETIO-s, ETIO-s, and ETIO-g compared to the vehicle blank (a vs. b, $P<0.001$).

3.2.2 Swimming Activity and Distance Travelled

When exposed to the steroid blend, RFs (n=8) increased the average movements by 11 and NRFs (n=8) increased the average movements by 12. RFs decreased the average distance travelled by 35.57 ± 7.29 cm and NRFs decreased the average distance travelled by 29.57 ± 8.42 cm (**Figure 19**). The average distance travelled for all females is significantly different from the vehicle blank ($H=10.886$, $P<0.05$). There was not a significant difference in average distance travelled between NRFs and RFs ($H=10.886$, $P>0.05$). This demonstrates that all females increased their activity over a smaller area of the tank in response to the blend of synthetic steroids.

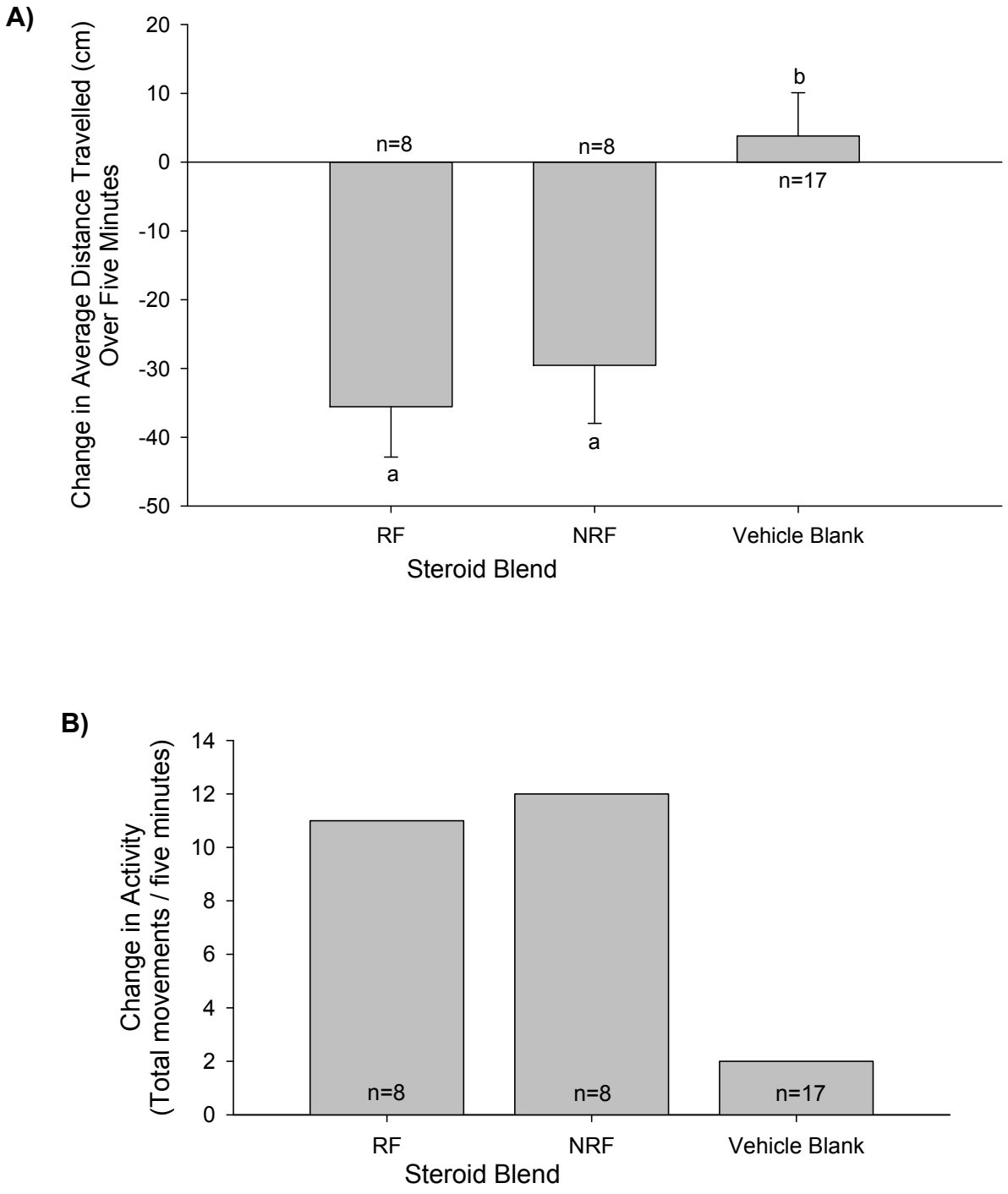

Figure 19. Both the RFs and NRFs decreased the average distance travelled (cm ± SEM) (**A**) and increased their total movements (**B**) after delivery of the synthetic steroid blend containing 11-oxo-ETIO, 11-oxo-ETIO-g, 11-oxo-ETIO-s, ETIO-s, and ETIO-g compared to the vehicle blank (a vs. b, $P<0.05$).

3.2.3 Gill Ventilation Responses

The synthetic steroid blend also elicited significantly different gill ventilation responses among the RFs (n=8, 13.5 ± 1.99 vent/min) and NRFs (n=8, 6.4 ± 0.51 vent/min) ($U=0.0$, $P<0.001$). Both were significantly higher than the vehicle blank ($U=0.0$, $P<0.001$) (**Figure 20**). This demonstrates that all females showed gill ventilation responses in response to the blend of synthetic steroids.

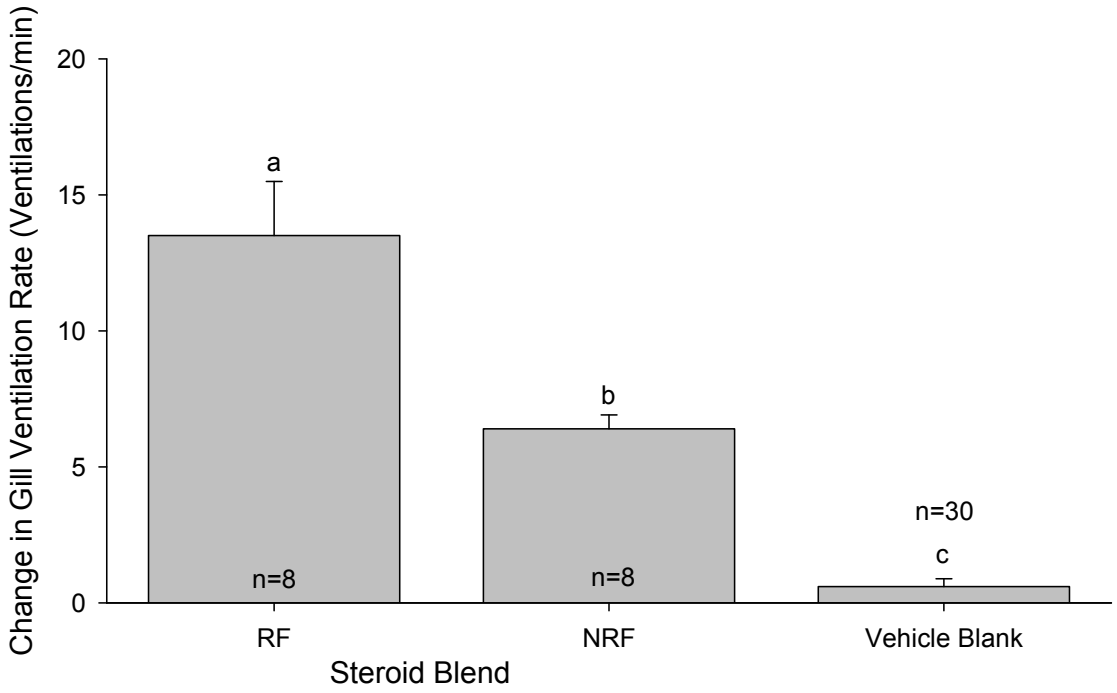

Figure 20. RFs increased gill ventilation rates (vent/min ± SEM) significantly higher than the NRFs ($P<0.001$) and the vehicle blank (negative control) ($P<0.001$). Bars with different letters are significantly different.

3.3 FEMALE RESPONSES TO URINE FROM GnRH-INJECTED MALES

3.3.1 Time Spent in the Inflow Zone

i. Urine from GnRH-Injected Males & Non-Urine Conditioned Water

When exposed to GnRH-injected RM urine diluted in background water by a factor of 10^4-10^5 (see section 2.2.2 for a detailed explanation of this dilution factor), both RFs (n=13) and NRFs (n=10) increased the time spent in inflow zone within the first five minutes after the onset of odour delivery (**Figure 21**). The RFs showed increases of 63.02 ± 4.68% and the NRFs showed 48.21 ± 7.81%, which were both significantly different from the vehicle blank ($F_{5,110}$=17.996, P<0.001) (**Figure 22**). There was no significant difference between responses of NRF and RF ($F_{3,67}$=1.529, P=0.448). This demonstrates attraction of all female round gobies to GnRH-injected male urine.

When exposed to the non-urine portion of GnRH-injected RM-conditioned water, both RFs (n=10) and NRFs (n=10) showed significant increases in the time spent in the inflow zone compared to the vehicle blank ($F_{5,110}$=17.996, P<0.001) (**Figure 21**). There was not a significant difference between responses of RFs (27.77 ± 8.79%) and NRFs (49.21 ± 9.38%) to non-urine conditioned water ($F_{3,67}$=1.529, P=0.117) (**Figure 22**). This suggests attraction of females to the non-urine conditioned water. Notably, there was a significant decrease among RFs when comparing urine to the non-urine conditioned water ($F_{3,67}$=1.529, P=0.001). Thus, for RFs, urine was more attractive than non-urine conditioned water, whereas urine was equally attractive as non-urine conditioned water for NRFs.

ii. Methanol Extracts & Aqueous Run-off of Urine

If the attractive compound or compounds in the urine are steroids, females should be attracted to the methanol extracts of urine, but not to the aqueous run-off from the solid phase extraction. Methanol extracts of urine elicited similar peak-recovery responses from females as urine (**Figure 21**). There was a significant difference in the change in time spent in the inflow zone in both RFs (n=10, 49.76 ± 5.79%) and NRFs (n=10, 40.56 ± 12.94%) compared to the vehicle blank ($F_{5,110}$=17.233, P<0.001), but not compared to each other ($F_{3,67}$=1.529, P=0.448), urine ($F_{3,67}$=15.971, P=0.183), or non-urine conditioned water ($F_{3,67}$=15.971, P=0.131) (**Figure 22**).

The aqueous phase from the MeOH extraction of the GnRH-injected RM urine was also delivered to determine if hydrophilic components of the urine were attractive. There was no significant difference in the responses of females between the aqueous run-off (n=10 RFs: 0.63 ± 8.28%, n=9 NRFs: 2.72 ± 7.38%) and the vehicle blank ($F_{5,110}$=17.233, P=0.184) (**Figure 22**).

The attraction to the methanol extracts and not to the aqueous run-off demonstrated that the attraction to the urine was transferred to the solid phase, not the aqueous run-off. This response also supports the hypothesis that the attractive compound or compounds are steroids.

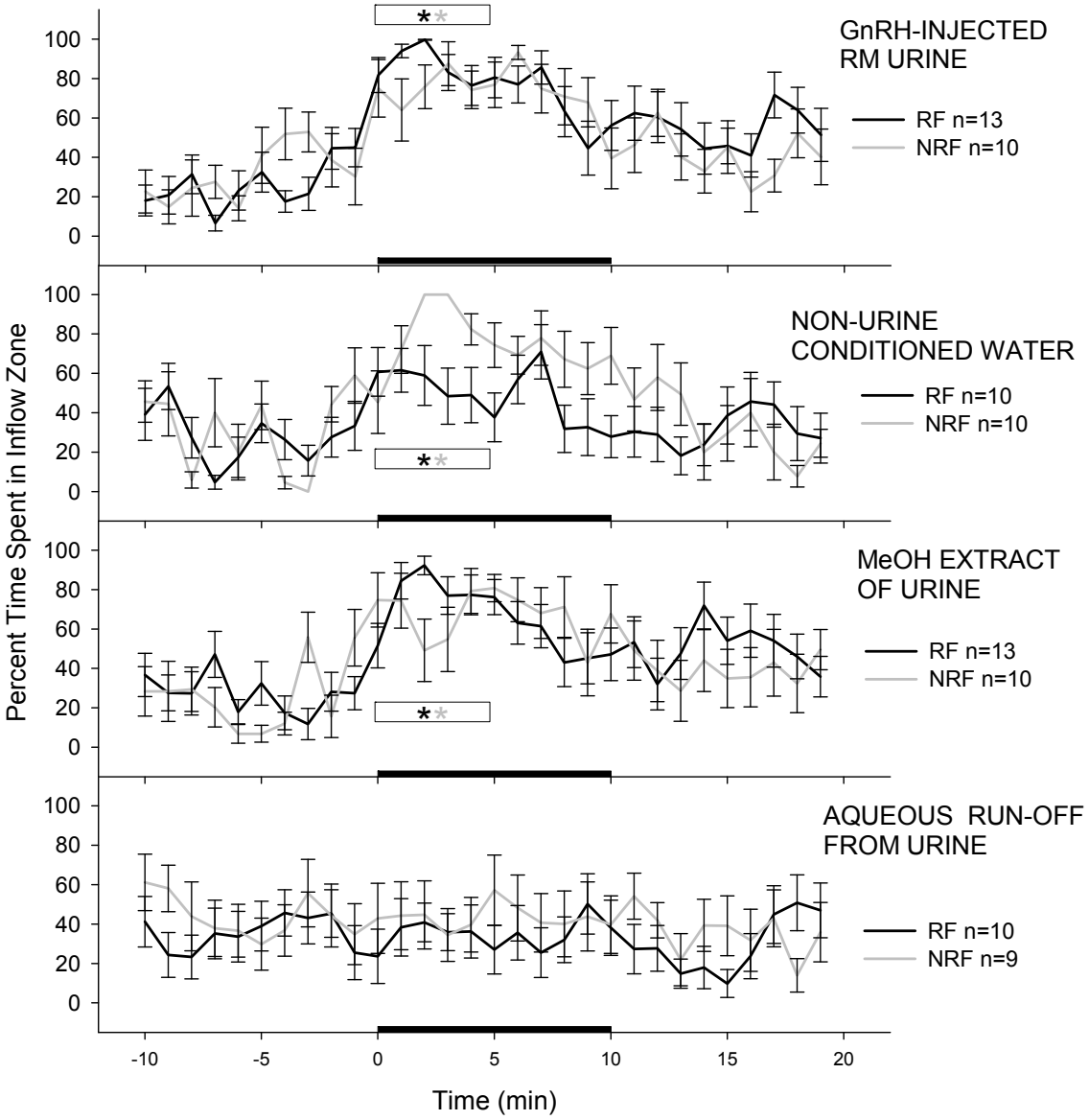

Figure 21. Average percent time (± SEM) spent in the inflow zone after delivery of GnRH-injected RM Urine, non-urine conditioned water, methanol extract of urine, and aqueous run-off of urine. The x-axis is time in minutes, with negative values referring to the pre-odour delivery period. Odour delivery period is denoted by the black bar on the x-axis. The boxed asterisk denotes significant difference over five minutes from baseline for each odour ($P<0.05$).

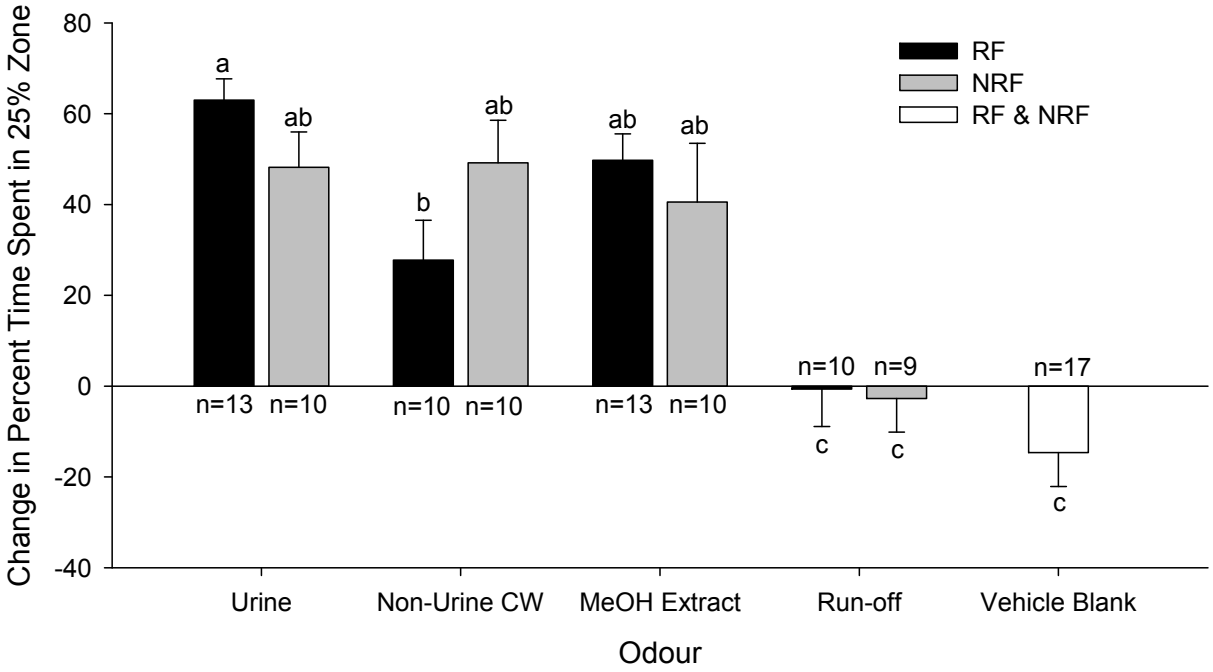

Figure 22. The average change in the percent time spent in the inflow zone (± SEM) after odour delivery increase significantly for GnRH-injected urine, non-urine conditioned water (non-urine CW), and methanol extract of urine (all P values <0.001). There was no significant difference between aqueous run-off of urine (run-off) and vehicle blank (P=0.184). Bars with different letters are significantly different.

3.3.2 Swimming Activity and Distance Travelled

As seen with food odour in this study, attractive urine odours should increase activity and decrease the total distance travelled. According to our hypothesis, if there is an attractive steroid in the urine, we should see this type of activity change during exposure to urine and the methanol extracts of urine.

i. Urine from GnRH-Injected Males & Non-Urine Conditioned Water

When exposed to GnRH-injected RM urine diluted in background water by a factor of 10^4-10^5 (to simulate natural dispersion conditions), females (n=23) increased the number of movements they made in the arena by an average of 10, and decreased the average distance travelled by 24.50 ± 3.27 cm (**Figure 23**). The average distance travelled was significantly different from the vehicle blank (n=17) (H=29.958, P<0.05). There was no difference in average distance travelled between NRFs (n=10) and RFs (n=13) (U=53.000, P=0.738). This demonstrates that both RFs and NRFs increased their activity and decreased the distance travelled in response to urine from GnRH-injected males.

In contrast, when the non-urine portion of GnRH-injected RM-conditioned water was applied to the tanks, females (n=20) increased their average movements by one after odour delivery and decreased the average distance travelled by 4.45 ± 4.08 cm (**Figure 23**). This was not significantly different from the vehicle blank (n=17) (H=29.958, P>0.05), and it was a significantly smaller decrease than urine (H=29.958, P<0.05). This suggests that there is a more pronounced change in swim activity of all females in response to GnRH-injected male urine than to the non-urine portion of GnRH-injected RM-conditioned water.

ii. Methanol Extracts & Aqueous Run-off of Urine

Methanol extracts of urine, when delivered to females (n=23), elicited an average increase of 5 movements, and a decrease in the total distance travelled of 12.10 ± 4.13 cm (**Figure 23**). This was significantly different from the vehicle blank (n=17) ($H=29.958$, $P<0.05$), but not from urine ($H_5=29.958$, $P>0.05$). This demonstrates that both RFs and NRFs increased their activity and a decreased the distance travelled in response to the methanol extracts.

The aqueous phase from the MeOH extraction of the GnRH-injected RM urine was also delivered to females (n=19). There was not a significant difference in the responses of females between the aqueous run-off (no change in movements, decrease in distance travelled of 4.03 ± 2.59 cm) and the vehicle blank (n=17) ($H=29.958$, $P>0.05$) (**Figure 23**). This demonstrates that neither RFs nor NRFs changed their swimming activity after delivery of the aqueous run-off.

These results show that the swimming responses seen to the urine was transferred to the solid phase, not the aqueous run-off. This supports the hypothesis that compounds responsible for female attraction to the GnRH-injected male urine are steroids.

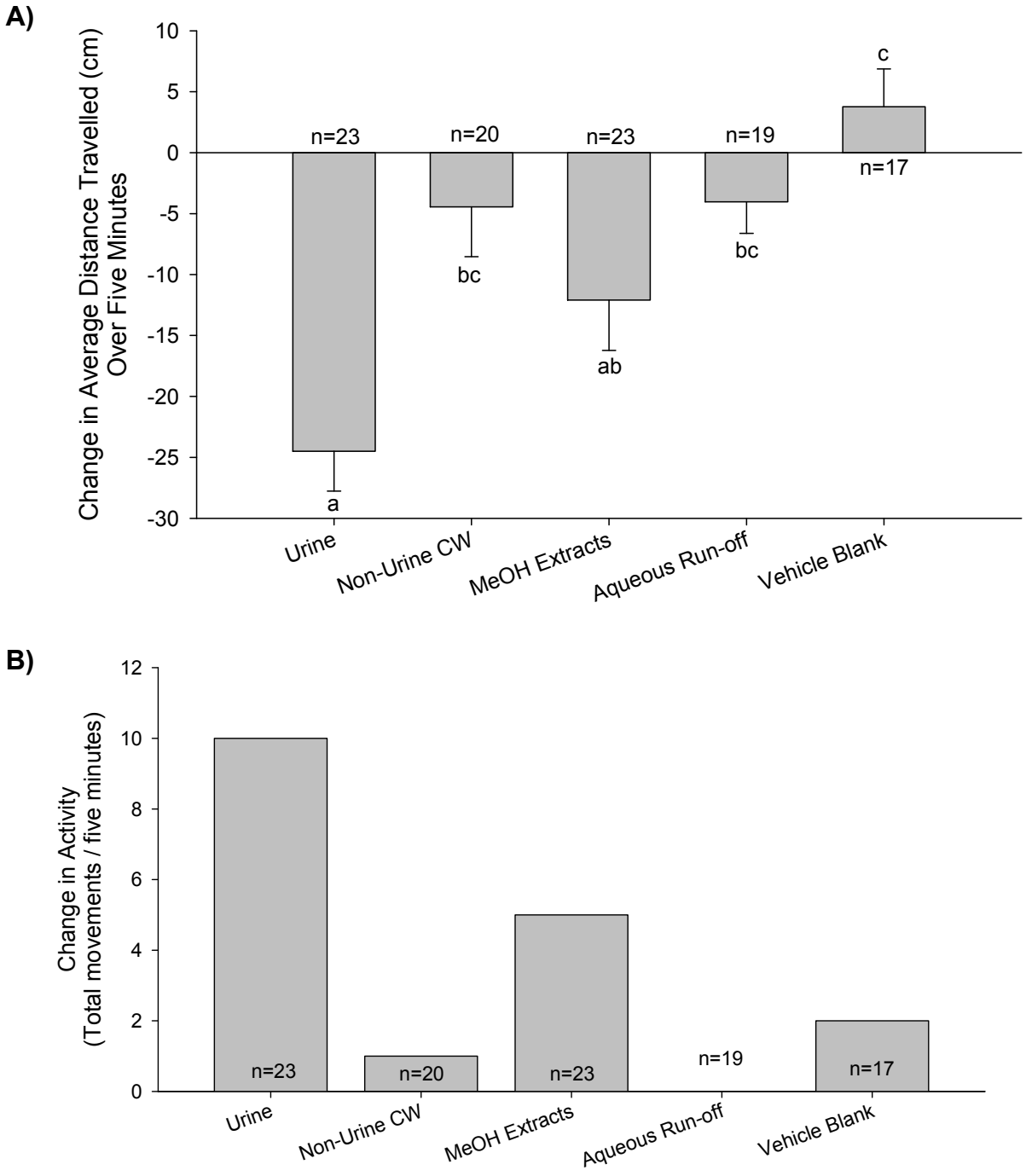

Figure 23. Average change in distance travelled (cm ± SEM) (**A**) and total number of movements (**B**) over the five minutes after odour delivery compared to the five minutes before odour delivery for urine, non-urine conditioned water (non-urine CW), methanol extracts of urine (MeOH extracts), and aqueous run-off of urine. RFs and NRFs were pooled since there were no significant differences for any odour. Note that the number of movements increase and the distance travelled significantly decreases compared to the vehicle blank (negative control) for urine ($P<0.05$) and the MeOH extracts ($P<0.05$). Bars with different letters are significantly different.

3.3.3 Gill Ventilation Responses

i. Urine from GnRH-Injected Males & Non-Urine Conditioned Water

When exposed to urine from GnRH-injected RM diluted by a factor of 10^4 - 10^5, both RFs (n=13) and NRFs (n=10) showed increases in gill ventilation after onset of odour delivery (**Figure 24**). Change in gill ventilation for NRFs of 6.71 ± 0.75 vent/min was significantly smaller than the change in gill ventilation for RFs of 11.09 ± 1.64 vent/min (U=106.0, P=0.012), and each group showed significantly larger changes than the vehicle blank (n=17) (H=76.285, P<0.05).

There were no significant differences in the change in female (n=20) gill ventilation rate between non-urine conditioned water (-0.56 ± 1.00 vent/min) and the vehicle blank (n=17) (H=76.285, P>0.05) (**Figure 24**), again suggesting that the females respond to the urine more than the non-urine conditioned water. This dataset substantiates the idea that the urine contains steroids because previous studies show increases in gill ventilation during steroid exposure.

ii. Methanol Extracts & Aqueous Run-off of Urine

When exposed to the methanol extracts of GnRH-injected RM urine, females (n=20) increased their gill ventilation rates by 6.83 ± 1.07 vent/min, which was significantly different from the vehicle blank (n=17) (H=76.285, P<0.05) (**Figure 24**). There was not a significant difference between the RF (n=10) and NRF (n=10) responses (U=56.5, P=0.110), nor was there a significant difference between the responses to methanol extracts and responses of the NRFs to urine (H=76.285, P>0.05). The responses to the methanol extracts were, however, significantly lower than the responses of RFs to urine (H=76.285, P<0.05).

However, the ability of the methanol extracts to elicit increases in gill ventilation supports the hypothesis that there is a steroid released in the urine.

There were no significant changes in the gill ventilation rate of females (n=20) in response to the aqueous run-off from the extraction of GnRH-injected RM urine (-0.33 ± 0.40 vent/min) compared to the vehicle blank ($H=76.285$, $P>0.05$) (**Figure 24**). This result was expected as there should not be steroids released in the aqueous run-off from the urine.

These data show that the responses to urine were transferred to the solid phase, not the aqueous run-off, when the urine was solid phase extracted and supports the hypothesis that there may be a steroid in the urine responsible for the responses seen to GnRH-injected RM urine.

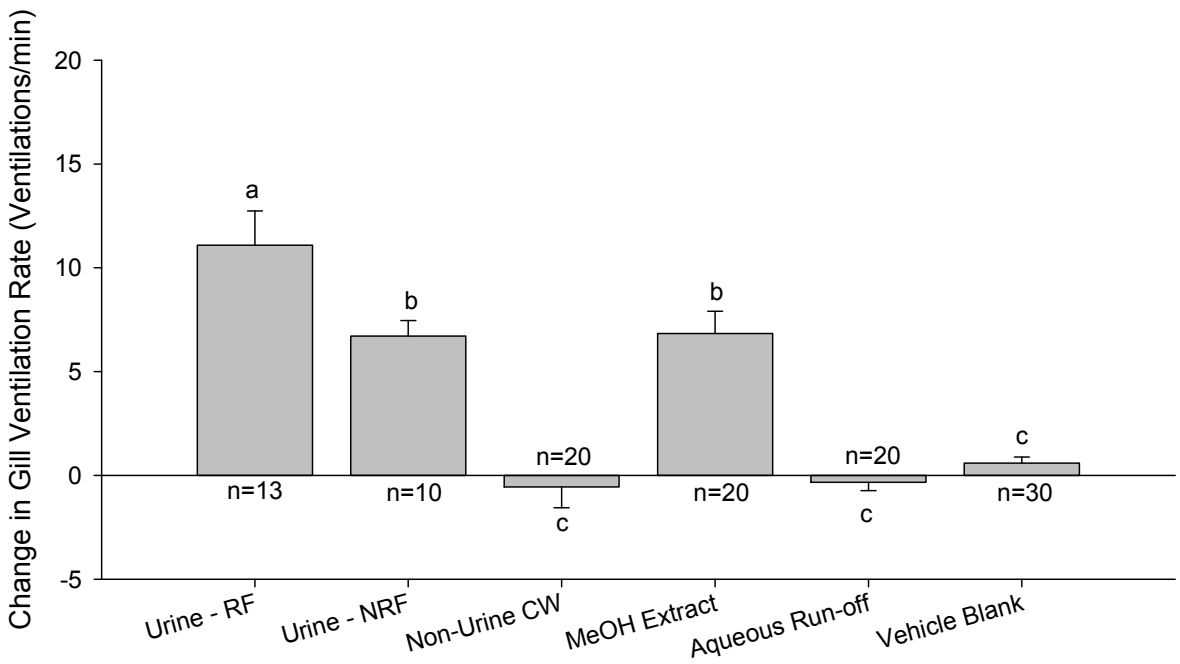

Figure 24. Average change in gill ventilation rates (ventilations/minute ± SEM) in the five minutes after odour delivery compared to the baseline ventilation rate for pooled RFs & NRFs. There was a significant increase in the gill ventilation rate after delivery of GnRH-injected urine ($P<0.05$) and methanol extract of urine ($P<0.05$) compared to the vehicle blank (negative control). Note that the reproductive female responses to urine were significantly higher than the non-reproductive female responses ($P=0.012$).

3.4 FEMALE RESPONSES TO HPLC-FRACTIONATED MALE-CONDITIONED WATER

The female round gobies were presented with pools of HPLC-fractionated whole GnRH-injected RM-conditioned water. Fraction pools are named according to the minutes they eluted from the column. It is important to note free 11-oxo-ETIO eluted between 26-31 minutes and the unknown conjugate of 11-oxo-ETIO eluted between 21-26 minutes. Pools of ten minutes were analyzed first, and any fraction pools that elicited strong attraction responses were further subdivided (see section 3.5 for the results from the subdivision).

3.4.1 Time Spent in the Inflow Zone

Of all the fraction pools tested, only fraction sets 21-31 and 31-40 elicited the attraction response-recovery trend exhibited from food odours (L-alanine) (**Figure 25**). Fractions 21-31 (n=8) caused an increase in time spent in the inflow zone of 33.18 ± 7.80% and fractions 31-40 (n=18) caused an increase of 13.10 ± 5.85% (**Figure 26**). Both of these fraction pools increased time spent in zone compared to the vehicle blank (21-31: $F_{10,130}$=8.735, P<0.001, 31-40: $F_{10,130}$=8.735, P=0.001). There were no significant differences between the responses of the RFs or NRFs for fractions 21-31 ($F_{10,130}$=8.735, P=0.244) or 31-40 ($F_{10,130}$=8.735, P=0.993). The responses to the vehicle blank were not significantly different from fractions 1-11 (n=18, $F_{10,130}$=8.735, P=0.168) or 11-21 (n=18, $F_{10,130}$=8.735, P=0.075). In addition, there was no significant difference in the change in time spent in the inflow zone after delivery of the aqueous run-off from the extraction of the fractions compared to the vehicle blank (n=13, $F_{10,130}$=8.735, P=0.079). This dataset suggests that there is attraction to fraction

pools 21-31 and, to a lesser degree, 31-40, but not to other fraction pools or the aqueous run-off.

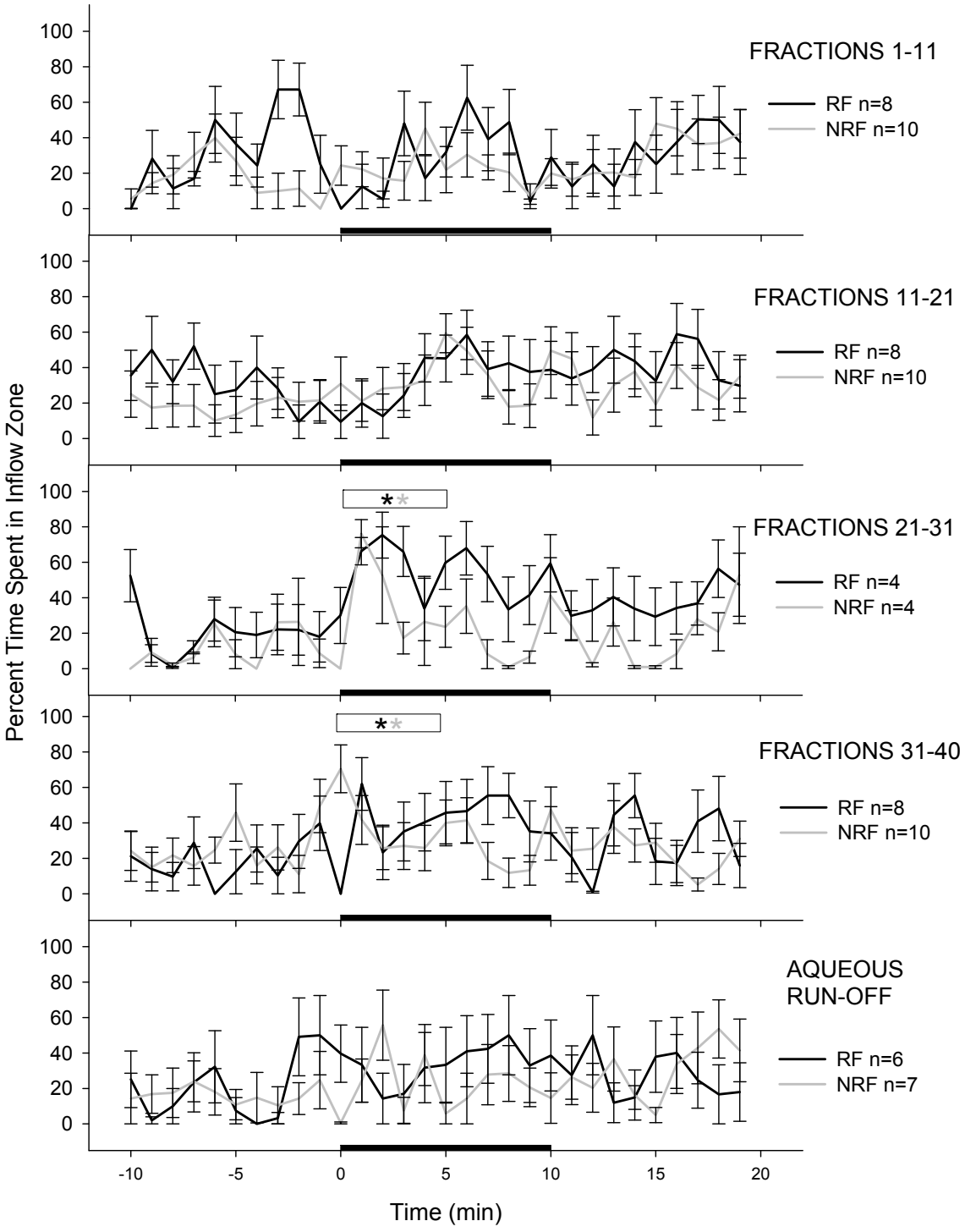

Figure 25. Average percent time (± SEM) spent in the inflow zone after delivery of fraction pools 1-11, 11-21, 21-31, and 31-40, and the aqueous run-off from extraction of the fractions. The x-axis is time in minutes, with negative values referring to the pre-odour delivery period. The black bar on the x-axis denotes odour delivery period. The boxed asterisks denote significant difference over five minutes from baseline for each odour ($P<0.05$).

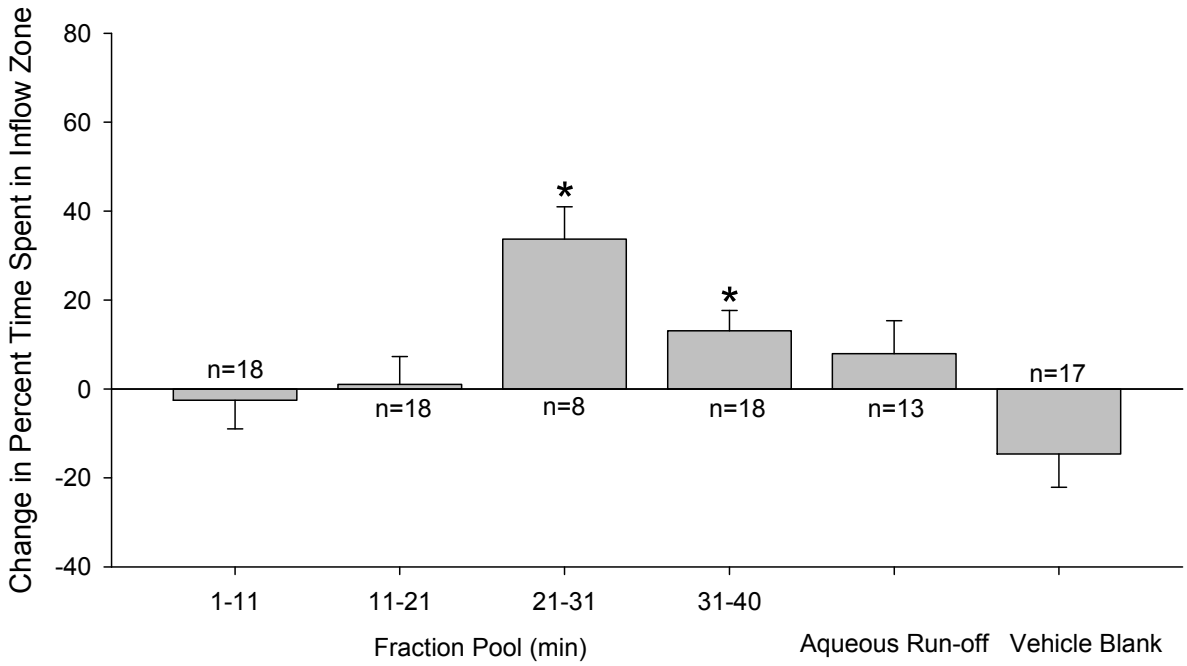

Figure 26. The average changes in the percent time spent in the inflow zone (± SEM) after odour delivery for fractions 21-31 and 31-40 are significantly increased compared to the vehicle blank (asterisks, $P(21\text{-}31)<0.001$, $P(31\text{-}40)=0.002$) whereas fractions 1-11 ($P=0.168$), 11-21 ($P=0.075$), and the aqueous run-off from the extraction of fractions ($P=0.079$) do not show significant differences from the vehicle blank (negative control: reconstituted dechlorinated water). None of these odours elicited significantly different responses from RFs compared to NRFs ($P>0.05$), hence the responses for RFs & NRFs were pooled.

3.4.2 Swimming Activity and Distance Travelled

The fraction pools that were attractive to females may also elicit changes in the swimming activity similar to the food odour. In the case of the fraction pools, only fractions 21-31 (n=8) showed a significant decrease in the average distance travelled of 11.33 ± 7.29 cm ($H=153.99$, $P<0.05$) (**Figure 27**). Fractions 21-31 caused an average increase in the movements of 12 in the five minutes after odour delivery. There was not a significant difference in the distance travelled between RFs (n=4) and NRFs (n=4) for fractions 21-31 ($U=6.00$, $P=0.686$). None of the other fractions or the aqueous run-off from the fractions elicited significant decreases in the average distance travelled compared to the vehicle blank (n=17) (all $H=153.99$, $P>0.05$). These observations show that only the fraction set 21-31, which includes free 11-oxo-ETIO and its conjugates, caused any significant changes in swimming activity.

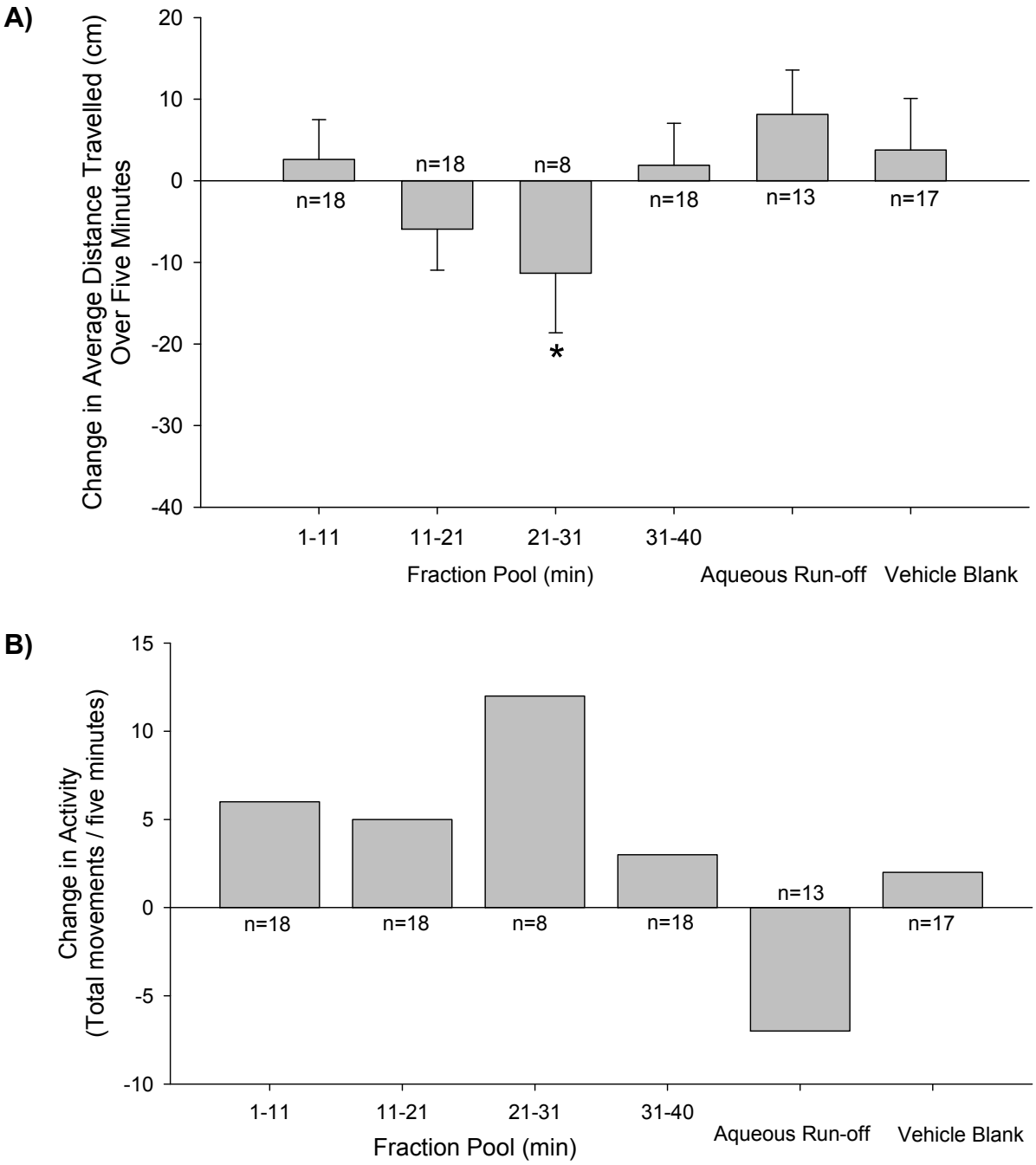

Figure 27. Average change in distance travelled (cm ± SEM) (**A**) and total number of movements (**B**) over the five minutes after odour delivery compared to the five minutes before odour delivery for fraction pools (in minutes) 1-11, 11-21, 21-31, and 31-40, as well as the aqueous run-off for pooled RFs & NRFs. Note that the number of movements increase and the distance travelled significantly decreases compared to the vehicle blank (negative control) for only fractions 21-31 (asterisk, $P<0.05$).

3.4.3 Gill Ventilation Responses

Fraction pool 11-21 elicited an increase in gill ventilation of 3.16 ± 0.46 vent/min and fraction pool 21-31 elicited an increase in gill ventilation of 9.10 ± 1.12 vent/min when delivered to females (n=10) (**Figure 28**). The increases in gill ventilation for these fractions were significantly different than the vehicle blank ($H=60.099$, $P<0.05$). There were no significant differences between the responses of RFs (11-21: n=10, 21-31: n=5) and NRFs (11-21: n=16, 21-31: n=5) for these two groups (11-21: $U=63.0$, $P=0.751$; 21-31: $U=13.0$, $P=0.564$). Fraction pools 1-11, 31-40, or the aqueous run-off from the fractions elicited no significant changes in gill ventilation compared to the vehicle blank (all $H=60.099$, $P>0.05$). This demonstrates that there may be a steroid released in fractions 11-21 and 21-31 that cause increases in gill ventilation. Fractions 21-31 include free 11-oxo-ETIO and its conjugates, so these steroids may be contributing to the increases in gill ventilation.

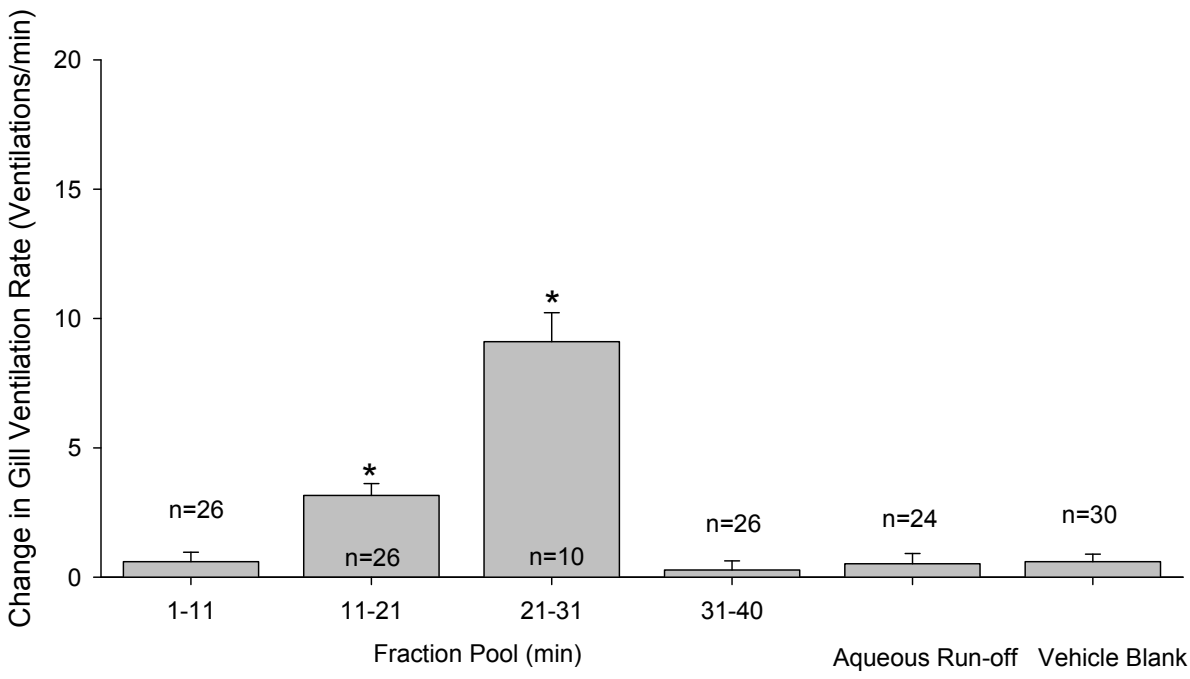

Figure 28. Average change in gill ventilation rates (ventilations/minute ± SEM) in the five minutes after odour delivery compared to the baseline ventilation rate for HPLC fractions eluting from 1-21 and 31-40 minutes as well as aqueous run-off from solid phase extraction of the fractions for pooled RFs & NRFs. Only fraction sets 11-21 and 21-31 (asterisks) showed significant increases in gill ventilation compared to the vehicle blank ($P<0.05$).

3.5 RESPONSES OF FEMALES TO POOLED FRACTIONS 21-26 AND 26-31 OF MALE-CONDITIONED WATER

Based on the attraction data (**Figure 26**), swimming data (**Figure 27**), and gill ventilation data (**Figure 28**) presented for this fraction set, fraction set 21-31 consistently showed positive behaviour responses for all three types of metrics taken. This supports the hypothesis that there may be a steroid released into the water that attracts reproductive females, but in order to progress towards the identification of the steroid or steroids responsible, further subdivision was required. Thus, we separated pool 21-31 into pools 21-26, which includes the unknown conjugate(s) of 11-oxo-ETIO, and 26-31, which contains free 11-oxo-ETIO. This subdivision allowed us to correlate the behavioural responses to fraction pools that contain specific steroids, although it is important to emphasize that these fraction pools may contain many other unknown compounds as well.

3.5.1 Time Spent in the Inflow Zone

i. Fractions 21-26 (Contains Unknown Conjugates of 11-oxo-ETIO)

Both RFs (n=8) and NRFs (n=10) showed a peak in time spent in the inflow zone within the first five minutes after odour delivery when given fraction pool 21-26, which contained the unknown conjugates of 11-oxo-ETIO (**Figure 29**). The RFs increased their time spent in the inflow zone by 59.90 ± 6.48%, which was significantly higher than the NRFs ($F_{7,109}$=4.456, P=0.010) (**Figure 30**). The NRFs increased their time spent in the inflow zone by 27.31 ± 8.42%. Both were significant increases from the vehicle blank (RF: $F_{10,130}$=8.735, P<0.001; NRF:

$F_{10,130}$=8.7351, P<0.001). This demonstrates that RFs were more attracted to the fractions 21-26, which include the unknown conjugate of 11-oxo-ETIO than NRFs.

ii. Fractions 26-31 (Contains Free 11-oxo-ETIO)

When presented with the fraction pool of GnRH-injected male-conditioned water that elutes from 26-31 minutes on HPLC (containing free 11-oxo-ETIO), differential RF and NRF responses were seen over the first five minutes after the onset of odour delivery (**Figure 29**). The NRFs (n=10) showed an increase of 33.44 ± 7.42% time spent in the inflow zone. This is significant compared to the vehicle blank ($F_{10,130}$=8.7351, P<0.001) (**Figure 30**). The RFs (n=8), however showed a decrease of -15.26 ± 8.39%; not significantly different from the vehicle blank ($F_{10,130}$=8.7351, P=0.952). This observation shows that only NRFs and not RFs are attracted to the fractions 26-31, which include free 11-oxo-ETIO.

iii. Olfactory Deprivation

Fractions 21-26 and 26-31 were also delivered to olfactory deprived fish to confirm that the responses seen were based purely on olfaction. We found this to be the case: neither of the fractions elicited any significant change in the time in inflow zone compared to baseline ($F_{3,20}$=2.560, P=0.084) (**Figure 30**).

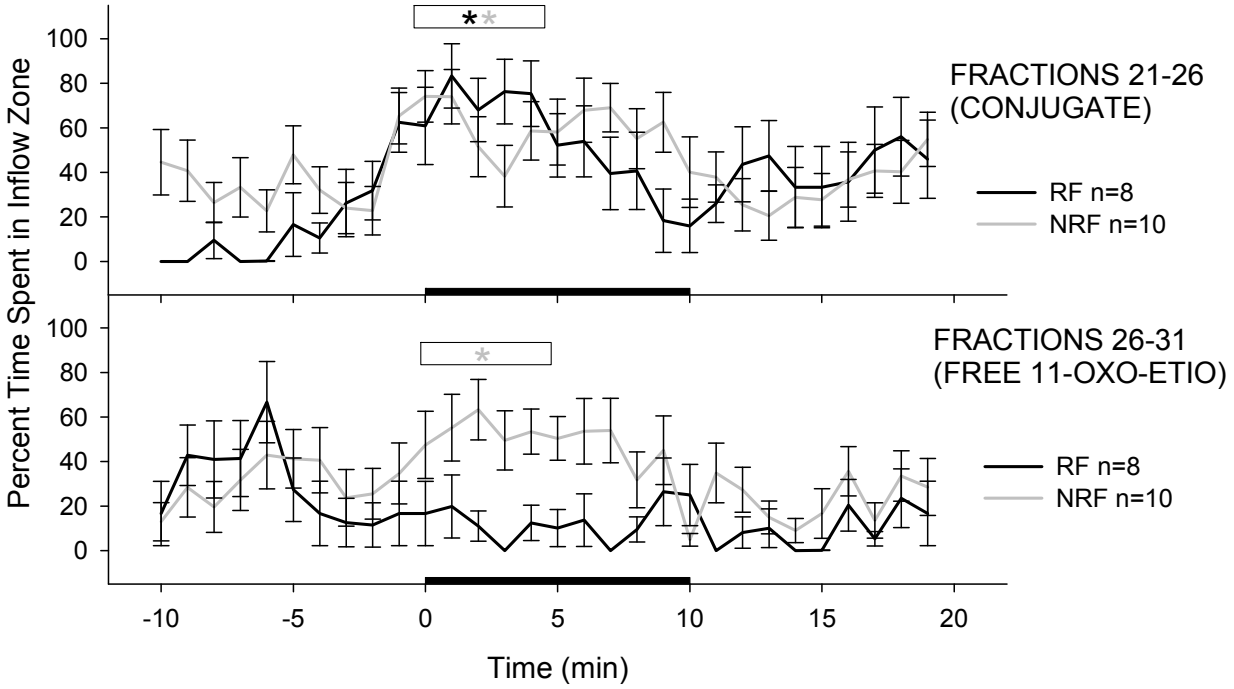

Figure 29. Average percent time (± SEM) spent in the inflow zone after delivery of fraction pools 21-26 (contains the unknown conjugate of 11-oxo-ETIO) and 26-31 (contains free 11-oxo-ETIO). All tests shown had peaks in the first five minutes after odour delivery (black bar) followed by recovery except fractions 26-31 delivered to RFs. The x-axis is time in minutes, with negative values referring to the pre-odour delivery period. The boxed asterisks denote significant difference over five minutes from baseline for each odour ($P<0.05$).

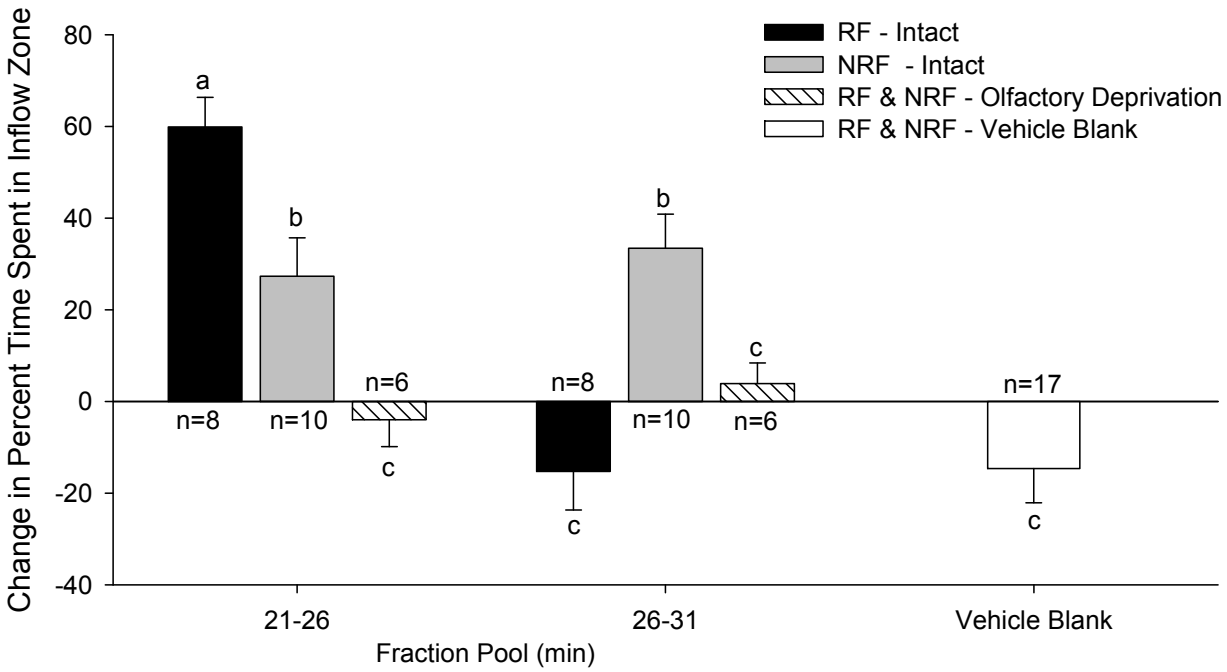

Figure 30 The average changes in the percent time spent in the inflow zone (± SEM) after odour delivery increase significantly for fractions 26-31 (contains free 11-oxo-ETIO) delivered to NRFs and fractions 21-26 (contains the unknown conjugate of 11-oxo-ETIO) delivered to both RF and NRF (all P values <0.001). There was also a significant difference between the RF and NRF responses within the fractions 21-26 group (P=0.010). There was no significant difference, however, between fractions 26-31 delivered to the RFs and the vehicle blank (P=0.952), nor was there a significant response to either odour in the olfactory deprivation trials (P=0.084). Bars with different letters are significantly different.

3.5.2 Swimming Activity and Distance Travelled

i. Fractions 21-26

The RFs (n=8) increased the movements by 10 and decreased the average distance travelled by 27.70 ± 7.29 cm and NRFs (n=10) increased the movements by 3 and decreased the average distance travelled by 5.19 ± 7.29 cm when fractions 21-26 (containing the unknown conjugate of 11-oxo-ETIO) were delivered (**Figure 31**). The responses of RFs were significantly different from the responses of NRFs ($t_{4,46}$=2.840, P=0.006) as well as the vehicle blank ($t_{4,46}$=4.630, P<0.001). The NRFs, however, were not significantly different from the vehicle blank ($t_{4,46}$=2.682, P=0.158). This demonstrates that both RFs and NRFs increase their swimming activity in response to fractions 21-26, which contains the unknown conjugate(s) of 11-oxo-ETIO, but only the RFs show a significant decrease in the distance travelled compared to the vehicle blank.

ii. Fractions 26-31

When presented with the fraction pool of GnRH-injected male-conditioned water that elutes from 26-31 minutes on HPLC (containing free 11-oxo-ETIO), the RFs (n=8) showed an decrease in movements of 3 and an increase in distance travelled of 13.66 ± 6.52 cm, which was not significantly different from the vehicle blank ($t_{4,46}$=1.563, P=0.125) (**Figure 31**). The NRFs (n=10) showed an increase in movements of 3 and a decrease in distance travelled of -14.46 ± 7.29 cm; also not significantly different from the vehicle blank ($t_{4,46}$=2.682, P=0.101). These results show that neither RFs nor NRFs significantly changed their swimming activity after delivery of fractions 26-31, which includes free 11-oxo-ETIO.

iii. Olfactory Deprivation

Neither fractions 21-26 nor 26-31 elicited any significant change in swimming activity or distance travelled ($F_{3,20}$=2.155, P=0.125) when the nostrils were plugged (**Figure 31**), demonstrating the swimming responses to these fraction pools were purely olfactory-based responses.

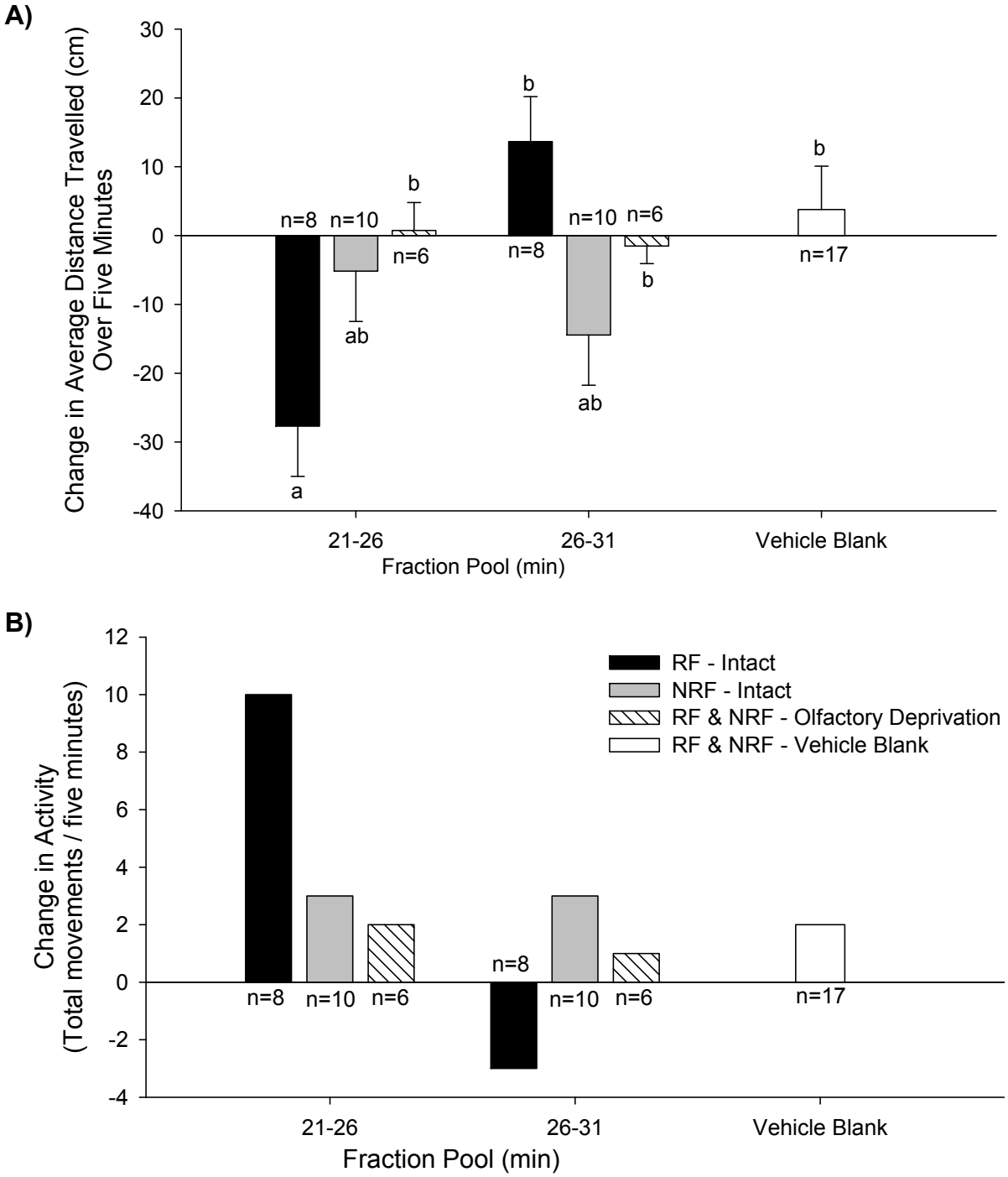

Figure 31. The change in average distance travelled (cm ± SEM) (**A**) and change in total movements (**B**) (five minutes after odour delivery minus five minutes before odour delivery) for fractions 21-26 (contains the unknown conjugate of 11-oxo-ETIO) and fractions 26-31 (contains free 11-oxo-ETIO). The movements increase and the total distance travelled decrease for fractions 21-26 delivered to RFs compared to the vehicle blank ($P<0.05$). None of the other odours tested here differed significantly from the vehicle blank (all P values >0.05). Bars with different letters are significantly different.

3.5.3 Gill Ventilation

i. Fractions 21-26

Gill ventilation responses to fractions 21-26 varied between RFs (n=10) and NRFs (n=16). The change in ventilation rate of the RFs (15.0 ± 1.16 vent/min) was significantly higher than the change of the NRFs (5.88 ± 0.78 vent/min) ($U=3.5$, $P<0.001$), both of which were statistically different from the vehicle blank ($H=63.746$, $P<0.05$) (**Figure 32**). This shows that both RFs and NRFs respond with increased gill ventilation to fractions 21-26 (includes the unknown conjugate of 11-oxo-ETIO).

ii. Fractions 26-31

When exposed to fractions 26-31 (contains free 11-oxo-ETIO), the RFs (n=10) increased gill ventilation rates by 12.13 ± 2.54 vent/min and the NRFs increased gill ventilation rates by 16.00 ± 2.12 vent/min (**Figure 32**). These increases were not statistically significant from each other ($U=96.0$, $P=0.092$), but they were statistically greater than the vehicle blank ($H=63.746$, $P<0.05$). The changes in the RFs were not significantly different ($U=108.0$, $P=0.539$), but the changes in the NRFs were significantly different ($U=350.5$, $P<0.001$) compared to the change in ventilation rate of females when delivered fractions 21-26.

These data show that females respond behaviourally with increased gill ventilation rates to both fraction pools 21-26, which includes the unknown conjugate of 11-oxo-ETIO, and 26-31, which includes free 11-oxo-ETIO.

iii. Olfactory Deprivation

Neither fraction pools 21-26 nor 26-31 elicited any significant change in gill ventilation rate ($F_{3,20}$=0.962, P=0.430) when the nostrils were plugged (**Figure 32**), demonstrating the gill ventilation responses to these fraction pools were olfactory-based responses.

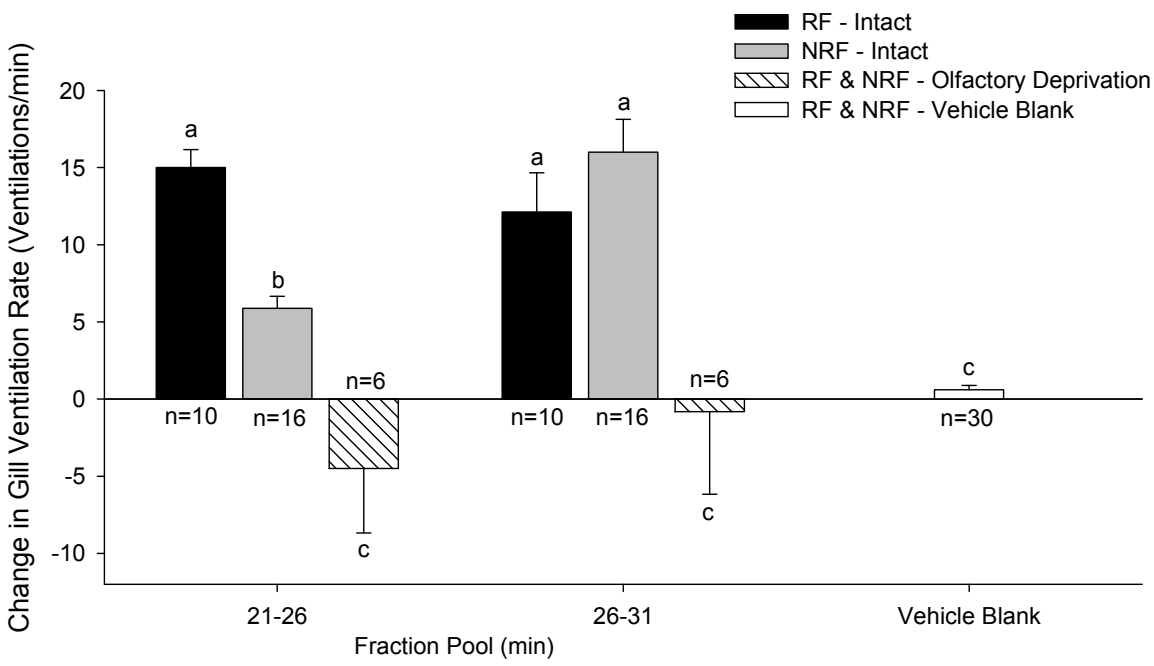

Figure 32. Average change in gill ventilation rates (ventilations/minute ± SEM) in the five minutes after odour delivery compared to the baseline ventilation rate for HPLC fractions eluting from 21-26 min (contains the unknown conjugate of 11-oxo-ETIO) and 26-31min (contains the free 11-oxo-ETIO) compared to the vehicle blank. All odours show significant increases compared to the vehicle blank (all P values <0.05), but there were significantly lower increases when NRFs were delivered fractions 21-26 compared to RFs (P<0.001). There were no significant responses to odours delivered in the olfactory deprivation trials (P>0.05). Bars with different letters are statistically different.

3.6 SUMMARY OF RESULTS

Table 2. Overall summary of the behavioural responses to each test odour, showing positive responses (++/+ indicates significance within each odour), no responses (-) and overall conclusions (attractive/not attractive).

Odour	Contents	Results			
		Time in Zone	Swim Activity	Gill Ventilation	Conclusion
Vehicle Blank	Food Odour trials: dechlorinated water Fraction trials: dechlorinated water run through methanol extraction Control for odour contamination, mechanical effects	-	-	-	Not attractive
Food Odour	10^{-5} M L-alanine in dechlorinated water - positive control – should elicit attraction in hungry females	+	+	+	Attractive to both RFs & NRFs
Synthetic Steroid Blend	Synthetic 11-oxo-ETIO, 11-oxo-ETIO-s, 11-oxo-ETIO-g, ETIO-s, and ETIO-g ethanol stocks diluted to 10^{-6} M in dechlorinated water	+	+	+	Attractive to both RFs & NRFs
Urine from GnRH-injected RMs	This urine contains conjugates of 11-oxo-ETIO, but not free 11-oxo-ETIO.	+	+	+	Attractive to both RFs & NRFs
Non-urine conditioned water	Mixture of substances (including free 11-oxo-ETIO) released from other parts of the body than by the urogenital papilla	RF+ NRF++	-	-	Weak attraction – more attraction in NRFs
Methanol extracts of urine	Solid phase from extraction of urine (non-polar compounds)	+	+	+	Attractive to both RFs & NRFs
Aqueous run-off from urine	Aqueous phase from extraction of urine (polar compounds)	-	-	-	Not attractive
Fraction Pool 1-11	Relatively polar molecules released	-	-	-	Not attractive

Odour	Contents	Results			
		Time in Zone	Swim Activity	Gill Ventilation	Conclusion
	into the methanol extracted and HPLC fractionated whole male-conditioned water				
Fraction Pool 11-21	Relatively polar molecules released into the methanol extracted and HPLC fractionated whole male-conditioned water	-	-	+ (weak)	Not attractive
Fraction Pool 21-31	Includes 11-oxo-ETIO and its conjugates	+	+	+	Attractive to both RFs & NRFs
Fraction Pool 21-26	Includes unknown conjugate(s) of 11-oxo-ETIO	RF ++ NRF +	RF + NRF -	RF ++ NRF +	More attractive to RFs than to NRFs
Fraction Pool 26-31	Includes free 11-oxo-ETIO	RF - NRF +	RF - NRF +	RF + NRF +	Not attractive to RFs; attractive to NRFs
Fraction Pool 31-40	Non-polar molecules released into the whole male-conditioned water	+ (weak)	-	-	Weak Attraction
Aqueous run-off from fractions	Aqueous phase from the extraction of the whole male-conditioned water (polar compounds)	-	-	-	Not attractive

4 DISCUSSION

This study used a novel high-throughput fractionation-attraction approach for isolating fish pheromones. This investigation includes the behavioural responses of RF and NRF round gobies to a blend of synthetic steroid representing compounds released by male round gobies; to urine from GnRH-injected RMs; and to fractionated extracts of male-conditioned water. The study fulfilled its specific objective of testing behavioural responses to synthetic steroids, of determining if GnRH-injected RM urine and methanol extracts of this urine are attractive to females, and of determining if pooled HPLC fractions of the GnRH injected-male conditioned water are attractive to females. Overall, the results supported the hypothesis that male round gobies attract females by releasing steroidal pheromones.

This study has expanded upon the findings of previous studies that have suggested that male round gobies release steroidal compounds that are readily detected by the olfactory epithelium of females (Belanger *et al.*, 2004), that RF gobies are attracted to an unknown chemical cue released by RM gobies (Gammon *et al.*, 2005), that 11-oxo-ETIO and conjugate forms increase gill ventilation rates in females (Murphy *et al.*, 2001), that RMs increase the number of urination events in the presence of females (Meunier & Corkum, 2007), and that RFs are attracted to RM urine (Yavno & Corkum, in preparation).

In this study, a combination of three behavioural measures was used: the time spent in the inflow zone, swimming activity, and gill ventilation rates. Attraction was said to occur when fish spent more time in the high concentration

zone, increased movements, and decreased distance travelled. Gill ventilation measurements were also taken to support the attraction responses, as previous studies have found increases in gill ventilation in response to steroids (Murphy *et al.*, 2001). Our findings with respect to these objectives are discussed in the subsequent subsections.

4.1 THE NOVEL HIGH-THROUGHPUT FRACTIONATION-ATTRACTION BEHAVIOURAL ASSAY IS EFFECTIVE FOR TESTING BEHAVIOURAL RESPONSES TO ODOURS

This high-throughput method, which allowed us to observe the behaviour of five fish simultaneously in response to small amounts of odour, has not been used in previous studies in the round goby. Similar high-throughput assay tank methods have been successfully used in examined responses to amino acids and food odours in zebrafish (Tierney *et al.*, 2008; Koide *et al.*, 2009). To our knowledge, this is the first such experiment that uses such an experimental design for identifying fish pheromones. Such an approach is useful when dealing with pheromones, as pheromones tend to have limited species overlap, whereas amino acids seem to act quite universally as attractive food odours in fishes (Sorensen & Stacey, 1999). A similar fractionation-attraction approach was successfully used in determining male responses to multi-component pheromones released by females (Linn & Roelofs, 1989). That study, however, used a one-at-a-time approach, rather than high-throughput.

Our assay relied on the diffusion of odour molecules in water, which led to the attraction responses to the inflow zone. Since different molecules have different diffusion properties in water, it is difficult to predict the exact diffusion patterns for all odours delivered, especially given that steroids are often non-polar (Fox & Whitesell, 2004). In theory, a non-polar molecule would not diffuse well in water, leading to the formation of micelles. However, the steroids we dealt with had conjugated groups, which confer polarity, thus increasing the solubility in water. It is also impossible to predict how the swimming of the fish itself will affect the diffusion of the odourant around the tank. Nevertheless, based on the results

from our food odour trials, it is clear that the high-throughput method allows the establishment of a concentration gradient in the tank.

As expected, this setup was free of delivery artifacts, as there was no change in the time spent in the inflow zone, swimming activity, or gill ventilation during the delivery of a vehicle blank of dechlorinated water. However, all three measures (time spent in the inflow zone, swimming activity and gill ventilation) pointed to behavioural responses during the application of the food odour, L-alanine. Previous studies in zebrafish have also shown that free amino acids, including L-alanine, are attractive to individuals that had not been fed prior to experimentation (Tierney *et al.*, 2008; Braubach *et al.*, 2009; Koide *et al.*, 2009). Thus, we continued these experiments on the assumption that pheromones will elicit similar attractive responses as the food odour.

It is important to note that fishes have mechanisms for chemoreception other than olfaction. For example, fish gustatory receptors are generally quite sensitive to free amino acids (Kasumyan & Dϕving, 2003). L-alanine is a palatable tastant (tastants were considered palatable if a flavoured pellet is not rejected by the majority of individual fish) to 12 of 14 species of fish examined in that study. We controlled for non-olfactory sensory modalities by performing these tests in fish that had undergone olfactory sensory deprivation. Neither the food odour nor the HPLC fraction pools elicited any significant behavioural responses, demonstrating that the behavioural responses tested in this study were purely olfactory-based. Thus, we can conclude that our assay was effective in determining whether or not odours were attractive to round gobies.

4.2 FEMALES ARE ATTRACTED TO A BLEND OF SYNTHETIC STEROIDS

We have shown that this high throughput assay indicates attraction to a blend of synthetic steroids, similar to a mixture previously investigated by Corkum *et al.,* (2008). We however, unlike Corkum *et al.*, excluded those free steroids which are not 5β-reduced (androstenedione, 11*β*-hydroxy-androstenedione, and 11-ketotestosterone) as well as one 5β-reduced steroid (ETIO, as it is only a minor product of round goby testes according to Arbuckle *et. al.*, 2005) from our blend, because Murphy et. al., 2001 have reported that only 5β-reduced steroids generate olfactory response and increase in gill ventilation in round goby females. The blend tested by the high-throughput approach included ETIO derivatives synthesized by round goby gonadal tissues (Arbuckle et al, 2005; Jasra, 2007), as well as the black goby pheromones (11-oxo-ETIO, 11-oxo-ETIO-s, 11-oxo-ETIO-g, ETIO-g, and ETIO-s). Males synthesize 11-oxo-ETIO, 11-oxo-ETIO-s and ETIO-s in the testes (Arbuckle *et al.*, 2005) and seminal vesicles (Jasra *et al.*, 2007), and there is evidence that males release 11-oxo-ETIO, 11-oxo-ETIO-s, and 11-oxo-ETIO-g (Katare *et al.*, in preparation). If this steroid blend contains the reproductive pheromone, it should be highly attractive to RFs. There were attraction responses to this steroid blend, suggesting that this blend of conjugated and unconjugated steroids can be used to test the attraction of both RFs and NRFs in the field.

Not all of these steroids may be released in significant quantities. ETIO-g, for example, is not synthesized by testes (Arbuckle *et al.*, 2005) or seminal vesicles (Jasra *et al.*, 2007) of the round goby. Belanger *et al.* (2004) found that, while there were EOG responses to fractions of HPLC-fractionated RM-

conditioned water containing 11-oxo-ETIO, ETIO, and ETIO-g, there was some unknown fraction that elicited much stronger EOG responses. Belanger *et al.* (2004) suggested that ETIO and ETIO-g do not likely play roles as pheromones, or else they should be potent odourants, which they are not. ETIO and ETIO-g, however, elicited gill ventilation responses in female round gobies (Murphy *et al.*, 2001). The results of our study clearly show that ETIO-g and ETIO-s can be combined with other steroids to form an attractive blend.

The results of this study suggest that we may be able to use a synthetic steroid analogue of the round goby reproductive pheromones to attract RFs and support the hypothesis that these steroids, which are released by male round gobies, attract females.

4.3 THE URINE OF REPRODUCTIVE MALES MAY CONTAIN A CONJUGATED STEROID THAT IS ATTRACTIVE TO FEMALES

In this study, both RFs and NRFs were attracted to urine from GnRH-injected RMs, as well as to the methanol extract of this urine and RFs showed more preference to the urine portion of the conditioned water than to the non-urine component. These findings support a previous study of female round goby attraction to RM urine (Yavno & Corkum, in preparation), and substantiate the hypothesis that RM urine contains a steroid that is highly attractive to RFs. Pheromonal steroids are released in the urine in other species. In another perciform fish, the Mozambique tilapia, for example, reproductive males urinate in pulses in the presence of gravid females for disseminating a sterol-like pheromone (Barata *et al.*, 2007; Barata *et al.*, 2008). In round gobies, reproductive males (but not non-reproductive males) increased urination pulses in the presence of females (Meunier & Corkum, 2007). If the compound responsible for the strong behavioural responses of female round gobies seen in male urine is indeed a steroid molecule, we would expect it to separate with the solid phase if the urine were to be solid phase extracted. We saw exactly that phenomenon: the females were attracted to the methanol extracts of urine, but not to the aqueous run-off. According to Scott & Vermeirssen (1994), sulfated steroids tend to be released in the urine. If this is the case, the attractive component in the urine may be a sulfated steroid.

4.4 REPRODUCTIVE FEMALES ARE ATTRACTED TO THE FRACTION POOL CONTAINING AN UNKNOWN CONJUGATE OF 11-OXO-ETIO, BUT NOT TO THE FRACTION POOL CONTAINING FREE 11-OXO-ETIO

When exposed to the fraction of male conditioned water containing the unknown conjugate of 11-oxo-ETIO (21-26 minutes), RFs showed very strong attraction responses. This is the first study aimed at progressing towards identifying the pheromone by narrowing down the fraction pools containing attractive compounds by testing behavioural or physiological responses to the HPLC fractions in the round goby. Such an HPLC fractionation approach has also been used to successfully identify putative pheromones in the sea lamprey (Li *et al.*, 2002; Yun *et al.*, 2003).

Arbuckle *et al.* (2005) found that 11-oxo-ETIO was the most abundant steroid produced on incubating round goby male testes with androstenedione (Arbuckle *et al.*, 2005). While the fraction pool containing free 11-oxo-ETIO attracted NRF, it failed to attract RFs. If 11-oxo-ETIO is the steroid used as the reproductive pheromone in the round goby, it should have attracted these females. Possibly, the 11-oxo-ETIO attracts NRF to the vicinity of the nests, so that these animals are already at the nest location during ovulation. Future studies could monitor ovulatory staging of the tested females.

The RFs were strongly attracted to the urine from GnRH-injected RMs. Biochemical analysis revealed that conjugated 11-oxo-ETIO, but not unconjugated 11-oxo-ETIO was present in this urine (Katare, in preparation). Further, the fraction pool containing the conjugated 11-oxo-ETIO was highly attractive to RFs. Thus, our results suggest that a conjugated form of 11-oxo-ETIO may be

responsible for the RF attraction responses, and consequently may be a reproductive pheromone.

Previous studies in the black goby (Colombo *et al.*, 1980) and round goby (Belanger *et al.*, 2004) have suggested that female fish may have status-dependent changes in olfactory sensitivity, so that females that are immediately ready to spawn are more strongly attracted to RMs than immature females. If the reproductive pheromones are indeed released in fraction pool 21-26, then our results suggest that there may also be status-dependent changes in behavioural responses in the round goby. This result further supports the hypothesis that the unknown conjugates released in fractions 21-26 may be used as reproductive pheromones in this species.

In contrast, only NRFs were attracted to the fraction pool containing free 11-oxo-ETIO. This finding corresponds to and expands upon the study done by Corkum *et al.* (2008), which found that a free steroid blend that included 11-oxo-ETIO non-significantly repelled RFs and significantly attracted NRFs. If RFs are repelled by free 11-oxo-ETIO, it is unlikely that RMs use free 11-oxo-ETIO to attract females to their nests to lay eggs. Rather, the attraction is observed in the fraction containing the unknown conjugate of 11-oxo-ETIO that is released in the male urine.

Increased gill ventilation in response to the fraction pool containing free 11-oxo-ETIO by both RFs and NRFs, substantiates the finding of Murphy *et al.* (2001) that both RFs and NRFs increased gill ventilation when presented with synthetic 11-oxo-ETIO. While both RFs and NRFs respond fraction pool 26-31 (containing 11-oxo-ETIO) by gill ventilation, only NRFs were attracted to this

fraction. Again, 11-oxo-ETIO may attract NRF to the vicinity of the nests, so that these animals are already at the nest location during ovulation, but more work needs to be done to substantiate this speculation.

These results encourage higher resolution behavioural testing of fraction pool 21-26 – in particular of fraction 24, which contains an immunoreactive conjugate (Katare, in prep.) Furthermore, the discovery of this highly attractive compound will have significant implications not only to the study of pheromonal communication in fishes, but also for a potential pheromonal control system for the round goby (reviewed in Corkum & Belanger, 2007). Pheromones are already used to control certain insect populations, and preliminary work is being done to design a trap system for the sea lamprey. In the sea lamprey, a specific bile acid was discovered to strongly attract ovulated females in the lab (Li *et al.*, 2002; Sorensen *et al.*, 2005a). A preliminary trap system baited with sperminating male lampreys was tested in the field, and it was found that nearly 74% of ovulated females released were actually caught by these baited traps (Johnson *et al.*, 2005). Later, a modified version of the trap using a synthetic version of the bile acid pheromone was tested in the field, and it was discovered that females will actually move upstream towards the traps (Johnson *et al.*, 2009). We may be able to apply the findings of this study to similar pheromone traps for the round goby.

5 CONCLUSIONS

The data presented in this thesis substantiate the chemical identification of sex pheromones in the round goby. This study is the first to use a high throughput fractionation-attraction approach to progress towards the identification of specific compounds that elicit behavioural responses in fish. This behavioural assay has proved to be a valuable tool for providing clues to the identity of the reproductive pheromone(s) in the round goby, and may be useful for other species as well.

Firstly, this study demonstrated attraction of both reproductive and non-reproductive females to a blend of synthetic steroids. This attraction response suggests effective use a blend of synthetic steroids analogous to those released by males, to attract reproductive females.

Secondly, this study demonstrated that both RF and NRF round gobies were attracted to urine from GnRH-injected RMs, and this attraction was transferred to the methanol extracts of this urine (which contains conjugated but not free 11-oxo-ETIO). This suggests that there may be a conjugated steroid released in the urine that is attractive to females.

Thirdly, this study showed that conjugated 11-oxo-ETIO may be responsible for the females' strong preference for urine from GnRH-injected males. This compound may be used in pheromonal communication between reproductive male and female round gobies.

Although these data are encouraging steps towards the development of a pheromonal control agent for the round goby, the exact structure of the molecule responsible for this attraction activity remains undetermined. Further chemical and

biochemical analysis partnered with behavioural analysis is required to determine the identity of the unknown conjugate.

This project was part of a collaborative project with participation by many contributing disciplines aimed at controlling the populations of the round goby in the Great Lakes. With further understanding of the pheromonal communication system in the round goby, conservation biologists may be able to develop feasible approaches to fisheries management in the Great Lakes and in other areas around the world threatened by the introduction of invasive fish species.

6 FUTURE STUDIES

This study provides a rapid behavioural assay for gaining insight into the attractive components of male urine in round gobies. The urine, and pooled fractions of methanol extracted conditioned water were tested, yet the exact chemical structure of the unknown conjugate is still undetermined. Firstly, the HPLC fractions can be further subdivided into smaller groups and purified to rule out effects of any other contaminant molecules in the fraction pools. These purified pools can then be tested for behavioural responses using this high throughput assay procedure.

Through the pairing of this rapid behavioural assay to such biochemical procedures such as mass spectroscopy, nuclear magnetic resonance spectroscopy, and affinity purification, it may be possible to determine how 11-oxo-ETIO is conjugated, and a synthetic analogue may be developed. Once a synthetic steroid analogue is developed, this behavioural assay may be used to test the attractive properties of the synthetic compound relatively quickly and effectively. There must not be any loss of attraction to the synthetic compound; otherwise its use as a control agent may not be effective.

Precedent for pheromones in fish population management is seen in the development of pheromonal traps for the sea lamprey, which required extensive field testing of a synthetic analogue (Johnson *et al.*, 2009). The sea lamprey studies support the use of a synthetic compound in similar traps placed in the field in order to summon gravid female round gobies into traps. Once such traps are developed, field testing will determined whether traps are a practical and

economical way of controlling round goby populations. Based on this study, it may be useful to set up traps that release a cocktail of attractive compounds, as it was found that a synthetic steroid blend containing a mixture of conjugated and unconjugated compounds was attractive to both reproductive and non-reproductive females. Given the growth of the round goby population, and expansion to inland waterways, it may be useful to attract as many fish as possible regardless of reproductive status.

Lastly, it may be useful to test behavioural responses of males to compounds released from females, in order to complete the understanding of communication between sexes in the round goby. This could lead to the development of a universal round goby trap that releases various odours that are attractive to males and females to trap both sexes.

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APPENDIX A: EQUIPMENT USED

C18 Sep Pak®: Waters, Milford, MA
Ethovision® XT: version 5, Noldus, Leesburg, VA
GraphPad Prism® 5: GraphPad Software, La Jolla, CA
High Performance Liquid Chromatography (HPLC): ODS-M80 Column, Waters, Milford, MA
Infrared Cameras: Matco model AX-808C-SH, St. Laurent, QC
Masterflex Tubing: 14 Pt-cured silicone, Masterflex, Vernon Hills, IL
Peristaltic Pump: Cole-Parmer model #7519-06, Vernon Hills, IL
Pipetman® P1000®: Gilson, Middleton, WI
SigmaPlot® 10: Systat, San Jose, CA
SigmaStat® 3.5: Systat, San Jose, CA
Spectrophotometer: Agilent 8453, Santa Clara, CA
CentriVap®: Model # 7984010, Laconco, Kansas City, MO

APPENDIX B: CHEMICALS USED

L-alanine ($C_3H_7NO_2$), MW = 89.09 g/mol
Sigma-Aldrich, Oakville, ON
CAS #56-41-7

ETIO (etiocholanalone, 5β-androstan-3α-ol-17-one), MW = 290.4 g/mol
Steraloids, Inc., Newport, RI
CAS #53-42-9
Stock: 1 mg/mL in ethanol

ETIO-g (etiocholanolone-glucuronide, 5β-androstan-3α-ol-17-one glucosiduronate), MW = 466.56 g/mol
Steraloids, Inc., Newport, RI
CAS #3602-09-3
Stock: 1 mg/mL in ethanol

ETIO-s (etiocholanolone potassium sulfate, 5β-androstan-3α-ol-17-one potassium sulfate), MW = 408.59 g/mol
Steraloids, Inc., Newport, RI
Cat #A3638-000
Stock: 1 mg/mL in ethanol

11-oxo-ETIO (11-oxo-etiocholanolone, 3α-hydroxy-5β-androstan-11,17-dione), MW = 304.42 g/mol
Steraloids, Inc., Newport, RI
CAS #739-27-5
Stock: 1 mg/mL in ethanol

11-oxo-ETIO-g (11-oxo-etiocholanolone-glucuronide, 3α-hydroxy-5β-androstan-11,17-dione glucosiduronate), MW = 480.55 g/mol
Steraloids, Inc., Newport, RI
CAS #17181-16-7
Stock: 1 mg/mL in ethanol

11-oxo-ETIO-s (11-oxo-etiocholanolone potassium sulfate, 3α-hydroxy-5β-androstan-11,17-dione potassium sulfate), MW = 406.47 g/mol
Steraloids, Inc., Newport, RI
Cat #A3500-000
Stock: 1 mg/mL in ethanol

2-phenoxy ethanol, MW = 138.16 g/mol
Sigma-Aldrich, Oakville, ON
CAS #122-99-6

s-GnRH (*OvaRH*® salmonid gonadotropic releasing hormone)

Syndel Laboratories, Inc., Vancouver, BC
For injections: 0.7% v/v dissolved in 0.9% v/v NaCl (vehicle: ddH_2O)

MS-222 (Finquel®, 5% tricaine methane sulfonate)
Argent Chemical Lab, Redmond, WA

Reprosil® Dental Caulking (Vinyl Polysiloxane)
Dentsply Caulk, York, PA

Sodium Chloride (NaCl), MW = 58.44 g/mol
Sigma-Aldrich, Oakville, ON
CAS #7647-14-5

APPENDIX C: TEST ANIMAL INFORMATION

Table 3. Animal identification code, length, weight, GSI, and status of all male round gobies used for GnRH injections and odour collection

Animal Code	**Odours Collected** (urine/fractions)	**Weight (g)**	**GSI**	**Status** *R=reproductive N=non-reproductive*
GRM-080612-1	Urine	43.40	1.18	N
GRM-080612-2	Urine	44.87	1.98	R
GRM-080612-3	Urine	50.40	1.29	R
GRM-080612-4	Urine	36.90	2.06	R
GRM-080616-1	Urine	19.10	0.62	N
GNM-080616-2	Urine	22.30	0.98	N
GRM-080616-3	Urine	21.20	1.08	N
GNM-080616-4	Urine	15.00	0.27	N
GNM-080704-1	Urine	52.32	0.46	N
GRM-080704-2	Urine	17.00	1.70	R
GNM-080704-3	Urine	39.30	1.04	N
GNM-080704-4	Urine	29.00	0.25	N
GNM-080714-1	Urine	20.54	0.78	N
GRM-080714-2	Urine	17.27	2.03	R
GRM-080714-3	Urine	9.05	3.31	R
GRM-080714-4	Urine	11.34	1.59	R
GNM-080714-5	Urine	16.05	0.37	N
GNM-080714-6	Urine	21.72	0	N
GNM-080718-1	Urine	19.41	0.21	N
GRM-080718-2	Urine	20.82	1.68	R
GRM-080718-3	Urine	13.27	3.99	R
GNM-080718-4	Urine	40.94	0.07	N
GNM-080718-5	Urine	18.03	0.23	N
GNM-080718-6	Urine	43.08	0.20	N
GRM-080723-1	Urine	20.93	1.62	R
GRM-080723-2	Urine	55.58	1.98	R
GNM-080723-3	Urine	15.83	0.51	N
GNM-080723-4	Urine	25.16	0	N
GRM-080723-5	Urine	16.29	4.54	R
GNM-080723-6	Urine	28.26	0.04	N
GNM-080723-7	Urine	35.33	0.99	N
GNM-080723-8	Urine	19.98	0.35	N
GNM-080723-9	Urine	15.39	0.19	N
GNM-080805-1	Urine	9.03	0.25	N
GRM-080805-2	Urine	48.38	1.99	R
GRM-080805-3	Urine	22.47	2.26	R
GNM-080805-4	Urine	34.12	0.89	N
GNM-080805-5	Urine	18.87	0.69	N
GNM-080805-6	Urine	15.84	0.18	N

GGM-080512-1	Fractions	14.40	-	-
GGM-080512-2	Fractions	21.80	-	-
GGM-080512-3	Fractions	22.20	-	-
GGM-080512-4	Fractions	20.30	-	-
GGM-080512-5	Fractions	23.70	-	-
GGM-080512-6	Fractions	23.80	-	-
GGM-080512-7	Fractions	10.60	-	-
GGM-080512-8	Fractions	20.20	-	-
GGM-080512-9	Fractions	16.20	-	-
GGM-080512-10	Fractions	27.50	-	-
GGM-080522-1	Fractions	29.83	-	-
GGM-080522-2	Fractions	52.60	-	-
GGM-080522-3	Fractions	28.10	-	-
GGM-080522-4	Fractions	38.00	-	-
GGM-080522-5	Fractions	28.30	-	-
GGM-080522-6	Fractions	29.80	-	-
GGM-080522-7	Fractions	79.00	-	-
GGM-080522-8	Fractions	26.00	-	-
GGM-080522-9	Fractions	67.00	-	-
GGM-080522-10	Fractions	40.00	-	-
GGM-080522-11	Fractions	15.20	-	-
GGM-080522-12	Fractions	46.00	-	-
GGM-080522-13	Fractions	30.70	-	-
GGM-080522-14	Fractions	31.70	-	-
GGM-080522-15	Fractions	26.70	-	-
GGM-080522-16	Fractions	24.20	-	-
GGM-080522-17	Fractions	20.70	-	-
GGM-080522-18	Fractions	28.70	-	-
GGM-080522-19	Fractions	27.50	-	-
GGM-080522-20	Fractions	26.40	-	-
GGM-080602-1	Fractions	74.10	-	-
GGM-080602-2	Fractions	26.60	-	-
GGM-080602-3	Fractions	20.90	-	-
GGM-080602-4	Fractions	25.80	-	-
GGM-080602-5	Fractions	22.90	-	-
GGM-080602-6	Fractions	48.40	-	-
GGM-080602-7	Fractions	44.00	-	-
GGM-080602-8	Fractions	27.30	-	-
GGM-080602-9	Fractions	30.80	-	-
GGM-080602-10	Fractions	36.10	-	-
GGM-080602-11	Fractions	12.50	-	-
GGM-080602-12	Fractions	24.30	-	-
GGM-080602-13	Fractions	33.80	-	-

Table 4. Animal identification code, status, and length of all female round gobies used for behavioural testing

Animal Code	**Status** *R=reproductive* *N=non-reproductive*	**Total Length (cm)**
BNF-080625-1	N	8.1
BNF-080704-1	N	10.5
BNF-080704-2	N	9.0
BNF-080704-3	N	10.8
BNF-080715-1	N	7.5
BNF-080715-2	N	8.7
BNF-080724-1	N	12.6
BNF-080724-2	N	10.5
BNF-080724-3	N	11.1
BNF-080724-4	N	7.5
BNF-080808-1	N	9.0
BNF-080808-2	N	8.1
BNF-080808-R	N	9.6
BNF-080815-1	N	9.9
BNF-080815-2	N	8.1
BNF-080815-R	N	12
BNF-080830-1	N	11.1
BNF-080830-2	N	10.2
BNF-080904-1	N	8.7
BNF-080904-3	N	9.0
BNF-080904-R	N	10.8
BNF-080918-1	N	7.5
BNF-080918-2	N	8.7
BNF-080918-3	N	12.0
BNF-080918-4	N	7.5
BNF-080918-R	N	9.3
BNF-080927-1	N	10.3
BNF-080927-2	N	10.1
BNF-080927-R	N	9.8
BRF-080625-1	R	11.4
BRF-080625-2	R	11.1
BRF-080704-1	R	12.0
BRF-080704-2	R	11.1
BRF-080715-1	R	10.2
BRF-080715-2	R	8.7
BRF-080715-3	R	12.0
BRF-080801-1	R	12.1
BRF-080801-2	R	7.5
BRF-080801-3	R	10.5
BRF-080801-4	R	9.3
BRF-080801-5	R	7.5
BRF-080808-1	R	11.1
BRF-080808-2	R	11.2

BRF-080815-1	R	10.2
BRF-080815-2	R	10.8
BRF-080822-1	R	11.4
BRF-080822-2	R	12.6
BRF-080822-3	R	10.5
BRF-080830-1	R	13.5
BRF-080830-2	R	11.7
BRF-080830-R	R	10.2
BRF-080927-1	R	11.3
BRF-080927-2	R	10.2

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