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# Female Round Goby (Neogobius melanostomus) Movement Responses to Pheromones: An Investigation of Current Methods and Future Needs

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**Female Round Goby (*Neogobius melanostomus*) Movement Responses to Pheromones: An Investigation of Current Methods and Future Needs**

By

**Jennifer Lee Smith**

A Thesis
Submitted to the Faculty of Graduate Studies
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2014

**Female Round Goby (*Neogobius melanostomus*) Movement Responses to
Pheromones: An Investigation of Current Methods and Future Needs**


by


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January 15, 2014

DECLARATION OF CO-AUTHORSHIP

I hereby declare that this thesis incorporates material that is result of joint research, as follows: This thesis also incorporates the outcome of joint research undertaken in collaboration with Dr. Eric Clelland and Dr. Michelle Farwell under the supervision of Dr. Barbara Zielinski.  The collaboration is covered in Chapter III of the thesis. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author (myself), and the contribution of co-authors was primarily through the provision of analysis of steroid concentrations via enzyme-linked immunosorbent assays.

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ABSTRACT


The round goby (*Neogobius melanostomus*) is an invasive benthic fish species to the Laurentian Great Lakes. Earlier studies have suggested that reproductive male urine containing conjugated 3α-hydroxy-5β-androstane-11,17-dione (11-O-ETIO), including 11-O-ETiO-3-S (a potent odorant to gobies), attracts reproductive females.  However attraction to isolated or synthetic 11-O-ETIO-3-S has not been tested. This thesis investigates chemical attraction in the laboratory environment by examining the effect of (1) providing the female with shelter, (2) fractionated conditioned water containing 0.1 nM 11-O-ETIO derivatives on females without a shelter and (3) 1 uM synthetic 11-O-ETIO derivatives in arenas with a shelter. It was found that: shelters are important for studying chemoattraction, isolated derivatives of 11-O-ETIO delivered in the 0.1 nM range were not attractive to females, and some females were attracted to 1 uM synthetic 11-O-ETIO-3-s when a shelter was provided.  Further studies are required to establish if the released steroids, including 11-O-ETIO-3-s attract females.

DEDICATION

I dedicate this thesis to my family and to Lewis John Bryant for all of their love and support over the years.

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# LIST OF ABBREVIATIONS

**11-O-ETIO**: 3α-hydroxy-5β-androstane-11,17-dione

**11-O-ETIO-3-s**: 3α-hydroxy-5β-androstane-11,17-dione 3-sulfate

**11-O-ETIO-17-s**: 3α-hydroxy-5β-androstane-11,17-dione 17-sulfate

**11-O-ETIO-3-g:** 3α-hydroxy-5β-androstane-11,17-dione 3-glucosiduronate

**11-O-ETIO-17-g:** 3α-hydroxy-5β-androstane-11,17-dione 17-glucosiduronate

**CW:** Conditioned Water

**CW ex:** Sep-pak extracted RM Conditioned Water

**RM:** Reproductive Male

**NM:** Non-reproductive Male

**RF**: Reproductive Female

**NF:** Non-reproductive Female

**HPLC:** High Performance Liquid Chromatography

**EIA:** Enzyme-Linked Immunosorbant Assay

**EOG:** Electro-Olfactogram

**sGnRHa:** Salmon Gonadotropin Releasing Hormone

**CHAPTER I -GENERAL OVERVIEW**

Chemical communication in fishes contributes to how fish react behaviourally and physiologically to their environment. Fish rely on chemical cues for predator avoidance, shoaling, homing, and reproduction (Solomon, 1977). Among these chemical cues are pheromones, defined as 'an odour or mixture of odours released by the sender that evokes in the receiver(s) adaptive, specific, and species-typical response(s), the expression of which need not require prior learning or previous experience' (Sorensen and Stacey, 2004).

This thesis investigated the movement responses of female round gobies (*Neogobius melanostomus*) to two types of olfactory cues, food extract and reproductive pheromones. Round gobies are an invasive species to the Laurentian Great Lakes (Jude et al., 1992) and as such have encouraged a surge of studies on their life history (reviewed by Kornis et al., 2012). Many of the studies have focused on the release and reception of pheromones (reviewed in Chapter II), but they have varied greatly in how they test female responses, and some have yielded contradictory findings. Despite the amount of effort and number of studies on this subject, there is still much that is not known about reproductive communication in the round goby.

Chapter II of this thesis reviews 9 studies on female round goby behavioural responses to putative reproductive pheromones and other olfactory cues. Chapter II also includes an experimental investigation on the effect of providing a shelter on round goby movement responses. I discuss the importance of apparatus type, flow rate, tank enhancements, behavioural metrics, and sample size when designing a study to test olfactory-mediated behaviours in round goby. Each of these factors can greatly affect not only how the fish *receives* pheromones, but also how they *respond* to them (Vickers, 2000; Johnson and Li, 2010). It is

likely that the variance in these factors across the 9 studies is (in part) the cause of some of the contradictory results. In this review I attempt to identify optimal methods to use based on round goby behaviour in the wild and similar studies in other species, as well as provide suggestions for future studies.

Chapter III is an experimental study of fractionated and synthetic analogs of steroids released by reproductive male (RM) round gobies. The RMs release a 5β-reduced steroid: 3α-hydroxy-5β-androstane-11,17-dione (**11-O-ETIO**) as well as four derivatives of this compound, **11-O-ETIO-3-s**: 3α-hydroxy-5β-androstane-11,17-dione 3-sulfate, **11-O-ETIO-17-s:** 3α-hydroxy-5β-androstane-11,17-dione 17-sulfate, **11-O-ETIO-3-g:** 3α-hydroxy-5β-androstane-11,17-dione 3-glucosiduronate,**11-O-ETIO-17-g**: 3α-hydroxy-5β-androstane-11,17-dione 17-glucosiduronate (Arbuckle et al., 2005; Katare et al., 2011). The HPLC fractionated RM CW containing the conjugated steroids has been shown to be attractive to reproductive females (RF), whereas the unconjugated ('free') steroid is attractive to non-reproductive females (NF) (Tierney et al., 2013). 11-O-ETIO-3-s is physiologically detectable to females (Laframboise & Zielinski, 2011) and makes up a large portion of the pheromone released by males (Farwell, unpublished) suggesting that it may be important in reproductive signaling. The goal of Chapter III was to test for movement responses to the fractionated derivatives and synthetic analogs of 11-O-ETIO. In Experiment 1, changes in female activity in responses to mixtures of HPLC fractionated RM CW containing 0.1nM 11-O-ETIO and its derivatives were tested (Fig. 1.1). In Experiment 2, female responses to 1μM synthetic analogs of these steroids were tested, with a focus on the effects of 11-O-ETIO-3-s and the addition of tank

enhancements (shelter and gravel) (Fig. 1.1).  In this experiment, I added a PVC shelter

and gravel substrate to the tanks in an attempt to increase female response rate (see

Chapter II).  Overall this thesis provides the first review of olfactory-mediated

behaviours in the round goby and is the first to examine the effects of isolated

conjugates of 11-O-ETIO from RM CW on female activity levels.

**Experiment 1**
- Did not provide shelter or substrate

**Experiment 2**
- Provided tanks with a PVC shelter and gravel substrate

**1. RM CW Extract**
- 10nM 11-O-ETIO and its derivatives, as well as all other steroids released by reproductive males.
- (Tested in both experiments)

**3. Synthetic Analogs**
- 1μM synthetic analogs of 11-O-ETIO, as well as 11-O-ETIO-17-s, -3-s, and -3-g

**2. HPLC Fractioned RM CW Extract**
- 0.1nM 11-O-ETIO and its derivatives (11-O-ETIO-17-s, -3-s, -17-s, -3-g, and –17-g

**3a. 'All Steroids'**
- 1μM 11-O-ETIO as well as 11-O-ETIO- 3-s, -17-s, and -3-g

**3b. 'Minus –3-s'**
- 1μM 11-O-ETIO as well as 11-O-ETIO-17 -s, and -3-g

**3c. '3-s Alone**
- 1μM 11-O-ETIO- 3-s

**2a. Fractions 16, 19-31: 'Minus –17-s'**
- 0.1nM 11-O-ETIO as well as 11-O-ETIO- 3-s, -3-g, and –17-g

**2b. Fractions 16-21, 24-31: 'Minus –3-s'**
- 0.1nM 11-O-ETIO as well as 11-O-ETIO-17-s, -3-g, and –17-g

**2c. Fractions 16-24, 27-31: 'Minus –3 &17-g'**
- 0.1nM 11-O-ETIO as well as 11-O-ETIO- 3-s and -17-s

**2d. Fractions 16-26, 30-31: 'Minus free'**
- 0.1nM 11-O-ETIO- 3-s, -17-s, -3-g, and –17-g

**Figure 1.1** Flow chart describing each of test solutions delivered in Experiments 1 and 2 of Chapter III.  Experiment 1 utilized mixtures of HPLC fractionated RM CW containing 0.1nM 11-O-ETIO and its derivatives.  Experiment 2 utilized 1μM synthetic analogs of these steroids in tanks provided with a shelter and gravel.

**CHAPTER II- A REVIEW OF EXPERIMENTAL DESIGNS USED TO TEST OLFACTORY-MEDIATED BEHAVIOURS IN THE ROUND GOBY (*NEOGOBIUS MELANOSTOMUS)* AND AN EMPIRICAL STUDY ON THE USE OF SHELTERS**

**1. INTRODUCTION**

1.1 LAB STUDIES OF OLFACORY-MEDIATED BEHAVIOUR IN FISHES

Many novel approaches to studying olfactory-mediated fish behaviour in a laboratory setting have been developed, most of which are species and/or study specific (as reviewed by Sorensen, 2013). When designing an experiment to test for behavioural responses to an olfactory stimulus, there are many variables to consider. These include, but are not limited to, apparatus type and size (flumes, y-mazes, glass tanks, large arenas), tank enhancements (such as substrate and refuge), flow rate, acclimation times, duration of odour delivery, and circadian rhythm. It is also important to consider the effects of species of interest, the type of olfactory cue (alarm, reproductive, or food), and whether fish were domestic or wild caught on behavioural responses (as reviewed by Johnson & Li, 2010). These variables must be considered in order to design an experiment to best suit the objectives of the specific study. Unfortunately, even well designed laboratory experiments have difficulty replicating behaviours seen in the field and often studies performed in a lab yield contradictory results to those tested in the field (Johnson & Li, 2010). Johnson and Li (2010) suggest that environmental, social, and physiological context can modulate fish behavioural responses to olfactory cues and may lead to these conflicting results. These contexts can include factors such as physical structure of the aquatic environment, hydrodynamics, temporal variation, social context, experience, learning, physiological and developmental status, size, hunger, age,

and stress. Due to the complexity of an aquatic environment it can be difficult to design experiments that replicate what a fish would experience in nature.

This chapter includes a review of nine published lab studies examining olfactory-mediated behaviours (largely pheromone reproductive cues) in the round goby (Table 2.1); as well as an experiment that examines the effect of providing a shelter on movement responses to food extract. I describe the importance of apparatus type, flow rate, enhancements, behavioural metrics, and sample size when designing a laboratory experiment investigating movement responses to olfactory cues in the round goby using evidence from round gobies and a variety of other fish species.

1.2 ECOLOGY OF THE ROUND GOBY

Round gobies are an invasive benthic fish species native to the Black and Caspian Seas, and were discovered in the Detroit River in the early 1990s and have since spread to each of the Laurentian Great Lakes (Jude et al., 1992; Kornis et al., 2012). They are believed to have entered our freshwater system through the dumping of ballast water of Trans-Atlantic cargo ships (Jude et al., 1992). Since their appearance, the round goby has had detrimental effects on local ecology as it displaces native benthic fish species such as the mottled sculpin (*Cottius bairdi*) (Bergstrom and Mensinger, 2009; Dubs and Corkum, 1996; Janssen and Jude, 2001) and consumes the eggs of many important game and commercial fish species (Jude et al., 1995; Sterinhart et al., 2004). Because of this, many studies have attempted to find a method to control or eliminate this species

(reviewed by Kornis et al., 2012).  One method in particular focuses on the use species-specific odours to lure gobies into traps.

1.3 ROUND GOBY REPRODUCTIVE BEHAVIOUR

Male round gobies find and defend nesting sites, then attract females to these shelters using a combination of auditory, visual, and olfactory cues (Kornis et al., 2012). Once near the nest, it is believed that additional communication occurs, likely to assess reproductive status (Meunier et al., 2009).  Females will deposit their eggs on the roof of the nest and males will then fertilize the eggs and continue to provide the sole parental care through aeration (fanning) of the eggs, removing dead/diseased eggs, and defense from nest predators (Meunier et al., 2009; Kornis et al., 2012).  There has been only a single detailed documentation of one pair of gobies in a laboratory setting (Meunier et al., 2009) and many aspects of round goby reproduction are still poorly understood.

The pheromones that reproductive males release and how females respond to them have been studied in an attempt to understand round goby reproduction and to develop an effective population management strategy (Table 2.1).  It has been determined that males release a steroid compound, $3\alpha$-hydroxy-$5\beta$-androstane-11,17-dione (11-O-ETIO) as well as four derivatives of this compound, **11-O-ETIO-3-s**: $3\alpha$-hydroxy-$5\beta$-androstane-11,17-dione 3-sulfate, **11-O-ETIO-17-s:** $3\alpha$-hydroxy-$5\beta$-androstane-11,17-dione 17-sulfate, **11-O-ETIO-3-g:** $3\alpha$-hydroxy-$5\beta$-androstane-11,17-dione 3-glucosiduronate,**11-O-ETIO-17-g**: $3\alpha$-hydroxy-$5\beta$-androstane-11,17-dione 17-glucosiduronate (Arbuckle et al. 2005; Jasra et al. 2007; Katare et al., 2011).  Water

conditioned by males releasing these steroids, and isolates of this water have been shown to elicit attraction in females (Gammon et al., 2005, Tierney et al., 2013). In theory, females could be lured into traps baited with synthetic versions of these male reproductive pheromones (reviewed by Sorensen & Stacey, 2004). Despite the large number of studies on this subject (Table 2.1), we have still not linked the particular steroid constituents to round goby mate attraction.

1.4 OBJECTIVES:

The goal of this review is to highlight the different strategies used for studying round goby behavioural responses to olfactory cues in a laboratory setting and suggest methods for future studies. I also empirically tested the effects of tank enhancements (PVC shelter and gravel substrate) on female round goby responses to a food-related odour (fish flake conditioned water) as this had yet to be tested. This is the first comprehensive review of round goby behaviour in a lab setting and is fundamental for the successful development of a pheromone-based trapping strategy. In the following sections I describe the importance of: apparatus type and flow rate, tank enhancements, behavioural metrics, and sample sizes, in an attempt to provide suggestions for future studies on round goby behavioural responses to olfactory cues.

1.5 IMPORTANT CONSIDERATIONS FOR STUDYING ROUND GOBY OLFACTORY-MEDIATED BEHAVIOURS

**Tank Type**

A variety of apparati have been implemented to test round goby behaviour, ranging from 5L tanks to 1m long flumes, to y-mazes (Table 2.1). Most have been used in studies that have successfully yielded responses to olfactory cues; suggesting that the size and shape of the apparatus is unlikely to inhibit round goby behaviours. With that said, the dimensions and overall shape has varied, and is likely based on the questions being asked. For example, a y-maze style flume can be useful in choice experiments (Corkum et al., 2008), whereas small rectangular tanks may be beneficial for testing simple, quick responses and are also good for testing multiple odours in a short time period (Tierney et al., 2013). It is important to note that larger arenas such as flumes, (where swimming from one end to the other is tested), can be more explicit in showing attraction versus a smaller, square tank. In the flume, the fish must exert more energy, and travel greater distances to reach the odour and as such the response is less likely to occur by chance.

 **Flow Rate** More important than shape/dimensions of the apparatus is the movement of water through it (Vickers et al., 2000; Johnson and Li, 2010; Tierney et al., 2011). Hydrodynamics can greatly influence olfactory mediated behaviours due to its effects on the dispersion of an odour plume within the test arena and over time (reviewed in Johnson and Li, 2010). When placed in a flume under varying flow rates, round gobies show positive rheotaxis with a critical swimming speed of 231±0.07L/min (Tierney et al., 2011) meaning that flow rates should not exceed this value. When studying round goby responses to olfactory based sexual cues, it is important to consider flow rate, as males will actively fan their enlarged pectoral fins as a method of pheromone dispersion

(Meunier et al., 2009; Wantola et al., 2013). It has been shown that male fanning can produce flow rates between 130 to 220mL/min (Meunier et al., 2009; Wantola, 2013). When male conditioned water was delivered at both high and low flow rates (in terms of the typical male fanning rates), reproductive females showed a stronger response in the low flow conditions (Wantola, 2013). Previous studies on female round goby responses to reproductive odours used flow rates ranging from 0-60mL/min and still found responses (attraction) to the olfactory stimuli (see Table 2.1). These results suggest that flow rates should be kept below 200mL/min and that lower flow rates are more likely to elicit female attractive responses to reproductive odours.

To date, no studies have examined the typical flow rates that females are exposed to in the field during the reproductive season. Round gobies inhabit a large range of habitat types so it is likely that the background flow rates are highly variable but further studies are needed to confirm this. Water flow rate can greatly influence a fish's behaviour in response to an olfactory cue. For example, the sea lamprey (*Petromyzon marinus*) uses chemically mediated rheotaxis to orient itself within an odour plume of a migratory pheromone, and without flow, the response is terminated (Bjerselius et al., 2000). This could explain why gobies in tanks with no flow did not exhibit strong behavioural responses (Murphy, 1998). Alternatively, the common carp (*Cyprinius carpio*) requires still-to slowly flowing water in order to spawn (Sorensen, 2013). Exposure to natural stream flow rates can be important for rheotaxis in salmonids and assists in the orientation and navigation during migration (Stabell, 1984).

Overall, understanding the natural flow regimes fish are exposed to during reproduction is fundamental for optimizing behaviours in a laboratory setting.

**Tank Enhancements**

A variety of flumes and tanks have been used to test olfactory-mediated behaviours in the round goby (Table 2.1). One particular area of interest is the use of tank enhancements such as shelters and substrate in these experimental arenas. The use of environmental enrichments has been shown to increase the occurrence of natural behaviours such as exploration in harbor seals (*Phoca vitulina concolor*) and gray seals (*Halichoerus grypus*) (Hunter et al., 2002) and reproduction in a variety of taxa (reviewed by Carlstead & Shepherdson, 1994).

In the wild and in a lab setting, round gobies will seek out shelter (pers. observation), likely for refuge from predators. It has been demonstrated that tethered gobies in sandy open habitats were at a higher predation risk than those in sheltered habitats (cobble and boulder) (Belanger & Corkum, 2003) and gobies generally prefer rocky substrate over soft substrate (reviewed by Kornis et al., 2012). Because gobies are a benthic fish species, the type of substrate available may not only be important in their habitat preference but also in their behaviour. Substrate type has been shown to be an important factor in the facilitation of natural foraging behaviours in goldfish (*Carassius auratus*) (Smith & Gray, 2011) and may be of particular importance when studying benthic fish species as it can affect their comfort levels (Sorensen, 2014). Shelters may also serve as a visual cue that is required to elicit reproductive behaviours in females.

Goldfish deposit their eggs on/near underwater flora and as such will only spawn when a green visual cue is available (Dr. Norm Stacey, Pers. Comm.). In fishes that require a particular substrate type for reproduction, the availability of substrate in a lab setting can have a strong effect on chemically-mediated behaviours (reviewed in Johnson and Li, 2010). Because none of the existing round goby studies directly tests for the effects of environmental enrichments such as substrate or shelters, we performed a study using two different tank designs (see below).

**Behavioural Metrics**

Typically studies on round goby behavioural responses to both food related and reproductive olfactory cues have measured responses in time spent near the odour source or the distance/velocity travelled (see Table 2.1). These metrics are typically expressed as a change based on the time period directly before odour delivery ('pre-stimulus' period or acclimation period). This method is practical for round goby behaviour as it controls for the average or 'baseline' activity levels of each fish (which can be highly variable). Stereotyped, reproductive behaviours are typically used to assess pheromone-mediated behaviours. This has been well studied in goldfish (Sorensen et al., 1998). For carps, a variety of metrics are associated with arousal: locomotor activity, nudges, pushes, chasing, and feeding activity (aroused fish feed less) (Sorensen, 2013). In male gobies, several behaviours have been described such as colour change, barking, and fanning (Tavolga, 1956, *Gobius niger*; Meunier et al., 2009), but unfortunately female round gobies do not appear to exhibit any stereotyped

behaviours when in the presence of a reproductive male or when exposed to male pheromones or pre-recorded calls (Murphy et al., 1998; Rollo et al., 2007). Several behaviours have been described by Murphy (1998) including biting, head lifts, hopping, fanning, roll overs, and coughing, but none seemed to be associated with reproduction.

There have been very few well documented cases of round goby mate choice and reproduction in the lab or the field and as such it is possible that certain behaviours may be associated with reproduction, but more studies are required. Distance and velocity can be informative behavioural metrics as it is known that animals must move in order to locate an odour source (Vickers, 2000). Gobies utilize a burst-and-hold method of swimming (Tierney et al., 2011) and as such, typical measurements such as velocity and distance travelled can be more difficult to measure. When observed in the lab, gobies typically move about through the use of small 'hops' (movements less than one body length, both vertical and horizontal) (Murphy, 1998). Often gobies will hop vertically and do not actually displace themselves (distance moved value of 0) (pers. observation). Because of this it may be better to examine changes in activity level via hopping rates rather than distance or velocity, especially when using smaller tanks in which fish are not required to travel great distances. In addition, the studies reviewed here vary in how they assess time spent near an odour, particularly in the proportion of the tank that is marked as the 'inflow zone.' In order to quantify attraction, the fish should spend more time in the high concentration zone (Vickers, 2000), thus the inflow zone should be standardized as the portion of the tank with the highest concentration. The actual area of this 'zone' should vary with type and volume of the apparatus,

concentration delivered and the flow rate used.  In a few of the studies, the direction of

movement or 'pathway' was assessed, but neither showed strong changes when an

odour was applied.  This is contradictory to typical animal navigation in an odour plume

(Vickers, 2000).  It is possible that the flow rates used in the round goby studies were

not high enough to elicit directed movements toward the odour source.

It is important that future studies determine a suite of reliable behaviours that

can be used to quantify responses to olfactory cues.  It is also important to note how the

type of apparatus can affect the behavioural metrics that are being assessed.   In the

studies that used larger flumes and tanks (up to 1 m long), distance moved and velocity

were positively correlated with odour delivery ( Gammon et al., 2005; Kasurak et al.,

2012), whereas in smaller tanks (15 cm long), distance and velocity decreased in

response to odour delivery (Tierney et al., 2013).  This is likely due to restrictions of

movement in these smaller arenas.  When searching for the odour source, the fish does

not have to travel very far in 5L tank in comparison to a 1m flume.

**Sample Size**

Behavioural studies of wild-caught species often have small sampler sample sizes

(less than 10 individuals) (Bell et al., 2009).  This can be due to time constraints

(experiments may extend over hours or days) or difficulty obtaining individuals (rare or

protected species, live in remote areas, and small population sizes).  Natural variation

between and within individuals can increase the standard error of measurements of

behavioural metrics and can cause data to be distributed non-normally.  Each of these

factors together make it difficult for researchers to detect statistical significance.  This

lack of statistical significance does not necessarily mean there is no biological

significance (reviewed by Thomas & Juanes, 1996).  Statistical power (the probability of

finding a statistically significant result when one exists) can be used to link the ideas of

statistical and biological significance (Thomas & Juanes, 1996).  Power analysis can

determine if an experiment will be able to produce a statistically significant result if a

biologically significant difference actually exists (Thomas & Juanes, 1996).  In an

extensive review of statistical power in behavioural ecology encompassing 697 studies,

the average statistical power was much lower than the preferred value of 0.8 (Jennions

& Møller, 2003).  These authors suggest that the best way to avoid this problem is by

increasing the sample size.  Many of the round goby studies examined in this review

show large variation in their data, and typically their studies yield data that is non-

normal in its distribution.  Unfortunately, despite their large densities in the Great Lakes,

it can often be difficult to obtain a large sample size, especially when fish must be

chosen based on reproductive status.  This problem is likely do the lack of access to high

density areas or areas that can be seined (one of the most common collection methods).

Depending on the variables in question, researchers should assess variance and

determine proper sample sizes when designing experiments.  Based on previous

research and findings discussed in this review, a sample size greater than 10 should be

used in order to reach a statistical power of at least 0.8.

Table 1. Studies Examining Olfactoy-Mediatated Behaviours in the Round Goby (*Neogobius melanostomus*)

| Reference | Apparatus | Flow Rate (mL/min) | Acclimation (h) | Stimulus* | Stimulus Duration (min) | Behavioural Metrics | Sample Size | Conclusions |
|---|---|---|---|---|---|---|---|---|
| Kereliuk (2009) | Glass tank (5L) no enhancements | 5 | 24 | 1. $10^{-5}$ M L-alanine 2. RM Urine $10^{-5}$ M 3. Urine extract $<10^{-6}$ M 4. Conjugated 11-OE $10^{-8}$ M 5. Free 11-OE $10^{-8}$ M 6. Mix of synthetic steroids $10^{-6}$ M** | 10 | Time in inflow (25%) Velocity Distance Hopping Gill Vent. Rate | 7-15 | Increase time in inflow and gill vent. rate / Decrease in velocity and distance / Increase in hopping (high variation, not significant) |
| Kasurak (2010) | Flume (1m) 96L no enhancements | 160 | 5 | 1. RM call 2. CW extract $10^{-5}$ M | 10 | Time in Inflow | 8 | Increase in time spent in inflow, very low response rate (only responses in July) |
| Murphy (1998) | Glass tank (100L) with PVC shelter & gravel | none | 120 | 1. Brine shrimp washings 2. $10^{-2}$ M L-alanine 3. $10^{-3}$ M steroids ** | 34 | Gape Fanning Cough Head lift Roll Over Movement & Activity Hop Shelter entry/exit Gill vent. rate | 6 | Only found changes in gill ventilation, not in any other behaviour |
| Gammon et al., (2005) | Flume (20L) Clear top shelter | 6 | 0.5 | RM CW $\sim 10^{-5}$ M | 30 (used first 15) | Time in inflow (50%) Velocity Pathway | 4-5 | Increase in time spent in inflow and velocity, no changes in pathway |
| Yavno & Corkum (2010) | 1m flume (20L), shelter | 40-45 | 1 | RM urine (0.2 ml) NRM urine (0.2 ml) | 15 | Time spent in shelter | 7 | No change in time spent in shelter |

*Concentrations listed are of solutions before delivery

** For detailed stimulus lists, see appendix

Table 1. Continued

| Reference | Apparatus | Flow Rate (mL/min) | Acclimation (h) | Stimulus* | Stimulus Duration (min) | Behavioural Metrics | Sample Size | Conclusions |
|---|---|---|---|---|---|---|---|---|
| Corkum et al., (2008) | Y-Flume (19L) shelter | 60 | 0.5 | 1. Synthetic steroids $10^7$M** | 20 (used last 12) | Time in arms<br>Preference index<br>Velocity<br>Pathway | 8 | Increased time spent<br>Preference for some odours over others<br>Increased velocity |
| Yavno & Corkum (2011) | Flume (1m)2L no enhancements | 25 | 1 | 1. Goby egg washings<br>2. Trout egg washings | 15 | Time Spent in Inflow (40%) | 12 | Increased time spent in inflow |
| Marentette & Corkum (2008) | Glass tank (15L), shelter | 30-35 | 0.33 | 1. 1L RM CW<br>2. 1L NRM CW<br>3. 1L RF CW<br>4. 1L NF CW | 8 | Time spent in shelter<br>Time spent in inflow<br>Activity level<br>Head orientation | 8 | 61% of males never left the shelter<br>No change in time spent in near shelter<br>Fish were immobile >90% of the time |
| Belanger et al., (2007) | Glass tank (5.5L), shelter | NA | 16 | Estrone (concentration range $10^8$M-$10^{12}$M) | 30 | Gill vent. rate | 10 | Increase to all concentrations except $10^{12}$M |

**2. TESTING EFFECTS OF TANK EHANCEMENTS ON ROUND GOBY RESPONSES TO FOOD ODOURS**

In order to further test the effects of some of these factors (particularly enhancements and sample size) I applied fish flake conditioned water to female round gobies in tanks with either no enhancements (no shelter or gravel) and tanks equipped with gravel substrate and a PVC shelter. I predicted that more females would show increased movement and time spent near the odour source when in tanks with enhancements.

2.1 EXPERIMENTAL ANIMALS

For these experiments we used female round gobies caught via angling and seining from the Detroit River (Windsor, ON) from May to October 2011-2013. Prior to the experiments, fish were held in accordance with University of Windsor animal care guidelines, and experimental procedures conformed to the guidelines of the Canadian Council of Animal Care. Fish were housed in 205L, gravel-lined, aerated, flow-through tanks held at 18±1°C under a constant photoperiod of 16:8 (L:D). Fish were fed daily with Nutrafin® fish flakes (Tetramin, Inc.). Experiments took place between May to September (the typical round goby mating season) (MacInnis & Corkum, 2000). Fish were tested in the afternoon, and under a low-light setting in an attempt to mimic shallow water light conditions. On the day prior to the experiment, females were transferred from lab housing tanks and placed into an experimental tank. Test solutions

were delivered to the tank through a glass pipette adhered to the far wall of the tank via a peristaltic pump.  Fish were deprived of food for 24 hours prior to experimentation.

2.2 EXPERIMENTAL DESIGN:

**1. Tanks:** Experiments were run in glass tanks (8cm X15 cm X15 cm) filled with 4L of dechlorinated water.  Four tanks were used simultaneously, each visually isolated by wrapping the tanks in black plastic.

**2. Flow-rate:** Tanks were under constant flow through (130mL/min) of dechlorinated water (18°C ± 2), which has been shown to be attractive to female round gobies (Wantola, 2013).

**3. Tank Enhancements:** Fish were tested in one of two tank types.  One tank was void of any tank enhancements (no shelter or substrate) and the other was equipped with a grey PVC tube (2X1.5 inch) and white gravel substrate (Fig. 2.1).

**4. Behavioural Metrics:** We compared the effects of tank enhancements through the use of the following behavioural metrics: time spent in the PVC shelter, time spent in the inflow zone (25% of the tank closest to odour delivery source), and the hopping frequency (a movement less than one body length, including both vertical and horizontal movements) when exposed to either control water (10mL of background water) or food odour (10mL of dechlorinated water conditioned with 100g of Nutrafin® fish flakes).  We predicted that fish provided with tank enhancements (shelter and gravel) would exhibit stronger responses to a food odour than fish without these enhancements.  Based on previous studies, these responses would be seen in a greater

amount of time spent in the odour source and a larger hopping frequency (Table 2.1)

(Kereliuk, 2009).  Paired t-tests were used to test for differences between test solutions

(background water or fish flake water) within a tank type.  Student's t-tests were used

when comparing behaviours across tank types.  Wilcoxon signed rank, and Mann-

Whitney rank sum tests were used (respectively) for non-normally distributed data.

**5. Sample Size**:  When comparing the activity levels of round gobies when exposed to

either control water or food odours in different tank types, I used 7-31 females (per

treatment, see figures).  Samples sizes varied because data was compiled from previous

data (unpublished).  The statistical power of all tests performed in this study were above

0.8.

## A: No Shelter or Gravel



## B: Shelter & Gravel



**Figure 2.1** Experimental tank set up for tanks with no enhancements (A) and with enhancements (B). The unenhanced tanks were void of a shelter and gravel substrate, whereas enhanced tanks were equipped with gravel substrate and a PVC shelter. The shaded box on the left represents the 25% of the tank closest to the test solution source.

## 3. RESULTS

### 3.1 TIME SPENT IN SHELTER

In general, when provided with a PVC tube, female round gobies spend most if not all of their time within the shelter (applies to approximately80% of females) when no olfactory stimulus is present (Fig 2). When exposed to water conditioned with fish flakes (their typical food source in the lab) however, the percentage of fish that stay in the shelter drops to 45%, suggesting that when presented with chemical food cues, fish become more exploratory. This trend was not significant however ($p=0.06$).

### 3.2 TIME SPENT IN INFLOW ZONE (25% OF THE TANK)

I examined the change in percent time individuals spent in the inflow zone when exposed to fish flake water in tanks with either no shelter or gravel substrate or in tanks provided with a PVC shelter and gravel (Fig. 2.3). When no shelter or gravel were present, fish exhibited almost no change in mean time spent in the inflow zone ($\pm$SEM) ($0.03\%\pm0.07\%$) (Fig. 2.3A) whereas when the PVC shelter and gravel substrate were present, 3 out of the 15 fish showed an increase in time spent in time spent in the inflow zone (mean $\pm$SEM, $4.89\%\pm4.01\%$) (Fig. 2.3B). Overall fish spent significantly more time in the inflow zone in tanks with no shelter or gravel than in those provided with these enhancements ($p=0.003$) (Fig. 2.3C). This is likely due to the large amount of time fish spend in the shelter when one was provided. Again it can be seen that when a food odour is presented, fish presented with fish flake water exhibit a larger increase in time spent in the inflow zone when a shelter and gravel are present (Fig 2.3C).

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3.3 HOPPING BEHAVIOUR

I compared the baseline hopping rate of females when exposed to 10ml of dechlorinated water (control) across each tank type (without shelter or gravel, or with shelter and gravel) (Fig. 2.4).  Data was compiled from the last 5 minutes of the first acclimation period for all fish.  I found that individuals that were not provided with these enhancements show a wide range of hopping activity from no hopping to over 80 hops total performed over 5 minutes (Fig. 2.3A).  When a PVC shelter and gravel substrate are provided, fish show less variation in hopping rate over the five minutes, ranging from no hops to less than 20 hops (Fig. 2.4B).  The average hopping rate differs significantly between the two tank types ($p=0.036$) with tanks with no shelter exhibiting higher hopping rates (Fig. 2.5).   It is important to note however that the variation in hopping rate when no shelter or gravel are present was quite large (Fig. 2.5B) when compared to tanks with shelter and gravel.  This could be an issue when measuring statistical differences in hopping rate in experiments using these types of tanks.

I also examined the hopping frequency across these same treatments and found a similar pattern.  In general fish hopped more in tanks with no enhancements than fish that were provided with a PVC shelter and gravel.  Again I found that when presented with fish flake water, fish provided with a shelter and gravel exhibited a larger change in hopping frequency ($p=0.07$) than those without ($p=0.44$) (Fig. 2.6).  Due to large variation in hopping rates across females, I also calculated the hopping response magnitude by dividing the total number of hops performed over the 5 minute fish flake

application period by the total number during the 5 minute control period for tanks with

no shelter or gravel (Fig. 2.7A) and with a shelter and gravel (Fig. 2.7B).  I found no

significant differences between tank type (p=0.29) likely due to small sample size (Fig.

2.7C).  Although it does appear as though fish that increase hopping frequency in

response to fish flake conditioned water exhibited a greater magnitude of change when

no enhancements were available compared to fish provided with a shelter and gravel

(Fig. 2.7).  Based on the data presented in this study and in chapter 3, it is difficult to say

whether or not shelters are beneficial when studying round goby behaviour.  Based on

personal observation, detection of a 'positive' response to an olfactory cue is very clear

and simple when shelters are present, as the fish will make a straight path from the

shelter to the inflow zone during the period of high concentration and not during any

other time.
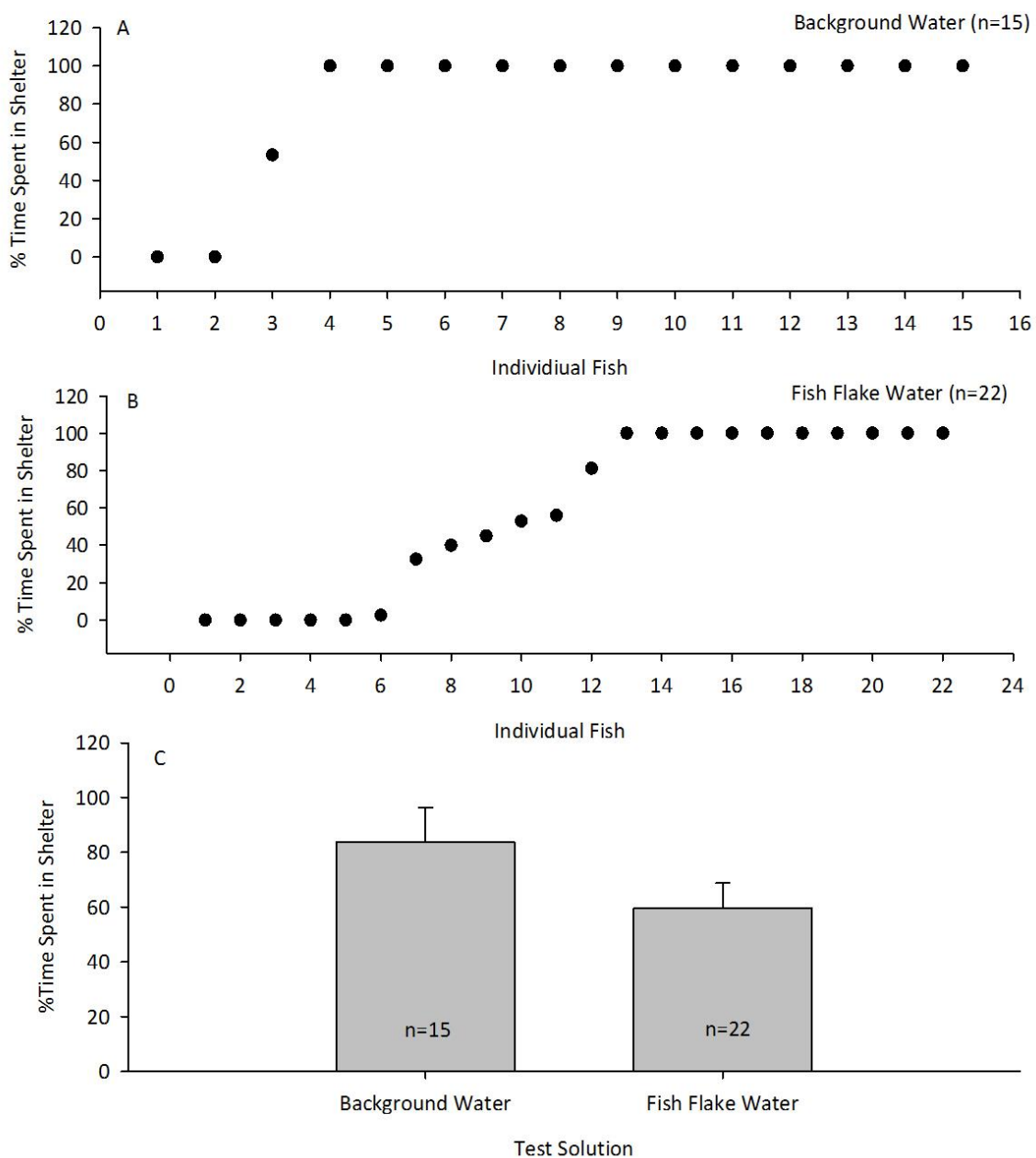
**Figure 2.2** Average percent time (per minute) (±SEM) each fish spent in the PVC shelter over 5 minutes when exposed to 10mL of background water (A) or fish flake water (B) (data sorted by y-axis values). Overall fish tended to spend less time in the PVC shelter when exposed to fish flake water (C), although not significant p=0.06.
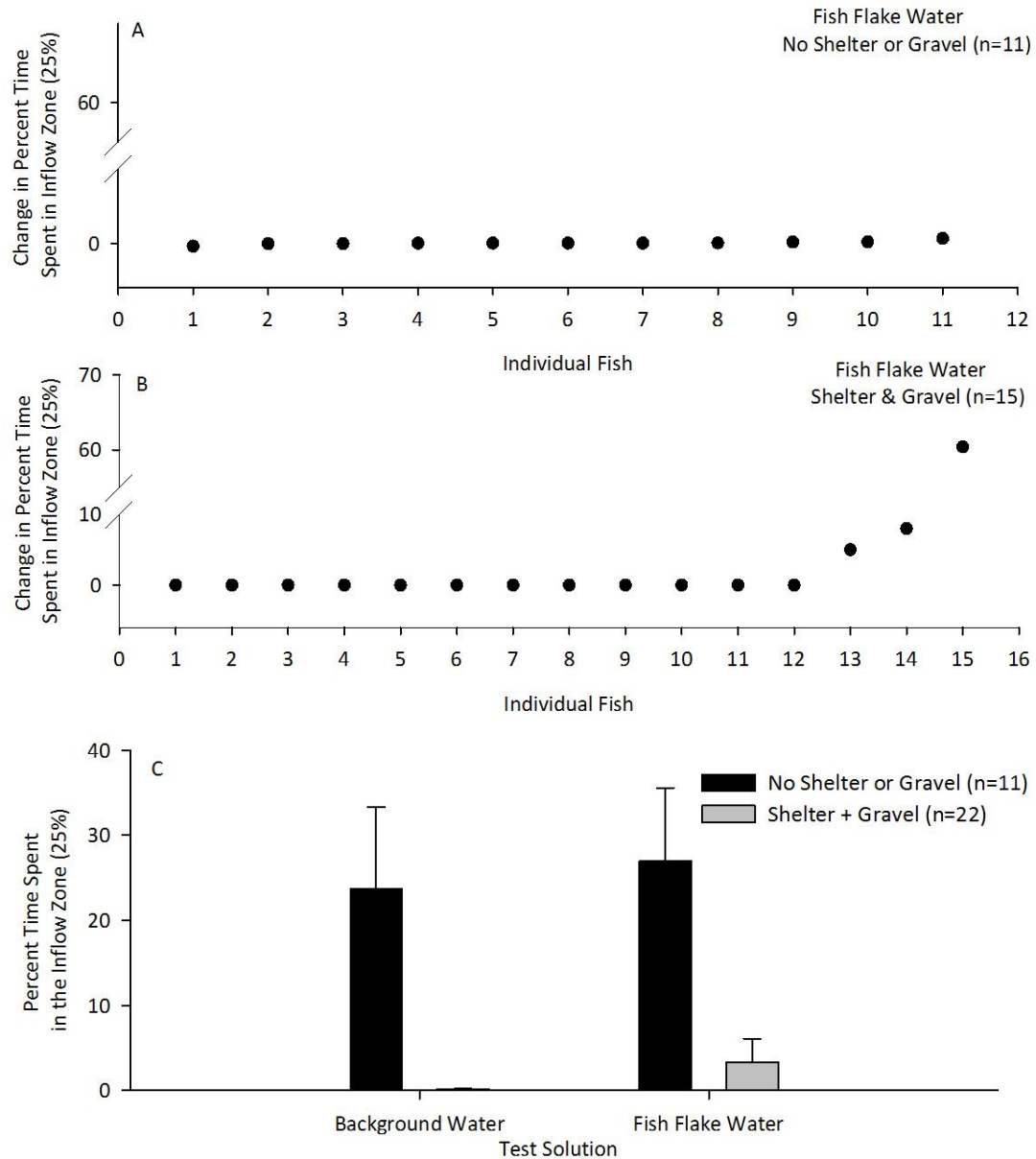
**Figure 2.3** Change in percent time spent individuals spent in the inflow zone (25%) of the tank when exposed to 10 mL of background water or 10mL fish flake water in tanks with either no shelter or gravel substrate (A) or tanks with a PVC shelter and gravel (B) over 5 minutes (data sorted by y-axis values). The average time spent in the inflow zone (±SEM) during delivery of each test solution in tanks with no shelter or gravel (black bars) and tanks with shelter and gravel (gray bars) is also expressed using a bar graph (C). There was no significant differences between time periods (within a tank type) (p=0.69 for no shelter or gravel and p=0.15 for shelter + gravel). When comparing across tanks with no shelter/ gravel and those with shelter and gravel, there was a significant difference (p=0.03).
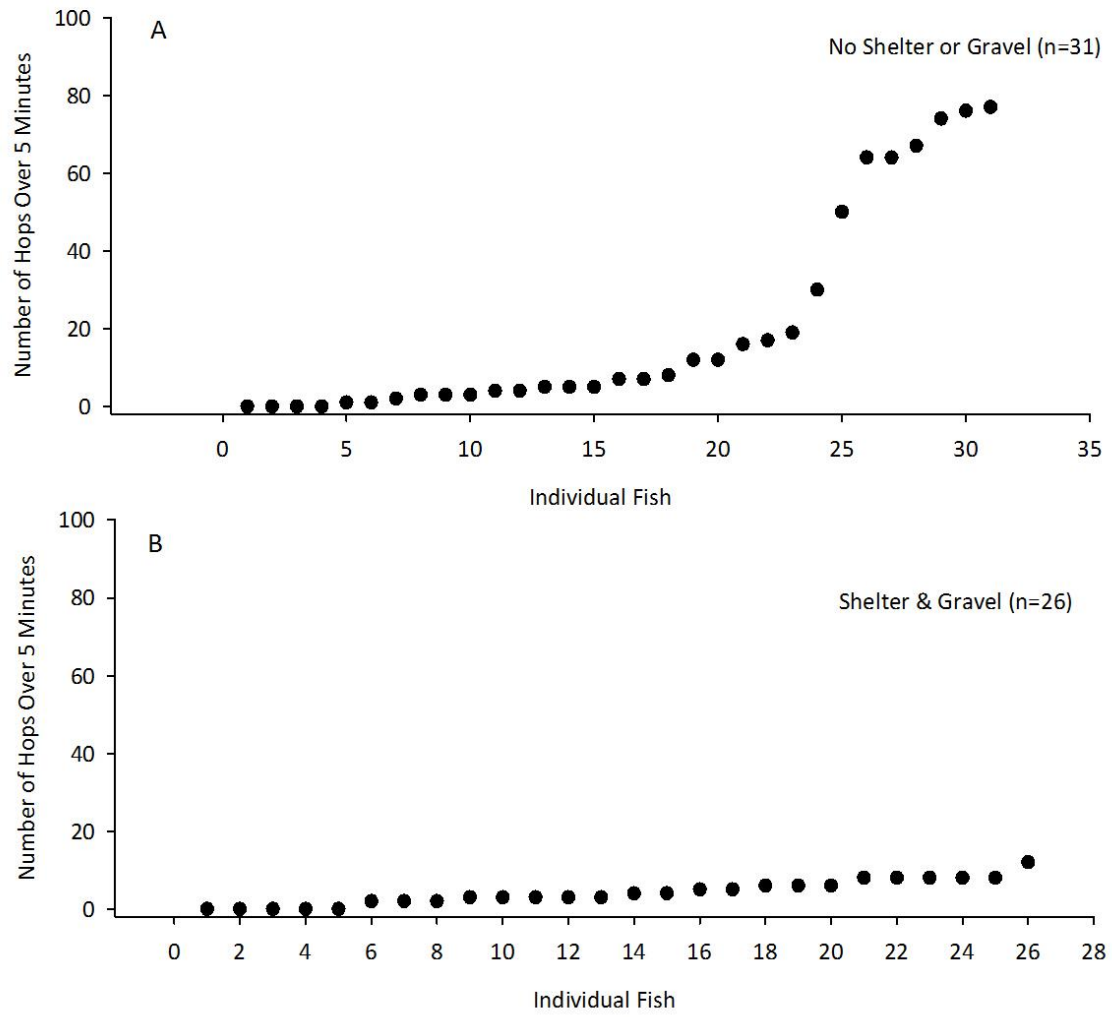
**Figure 2.4** Number of hops individuals performed over 5 minutes in after addition of 10 mL of background water in tanks without a shelter or gravel (A) and tanks with a PVC shelter and gravel substrate (B)(data sorted by y-axis values).
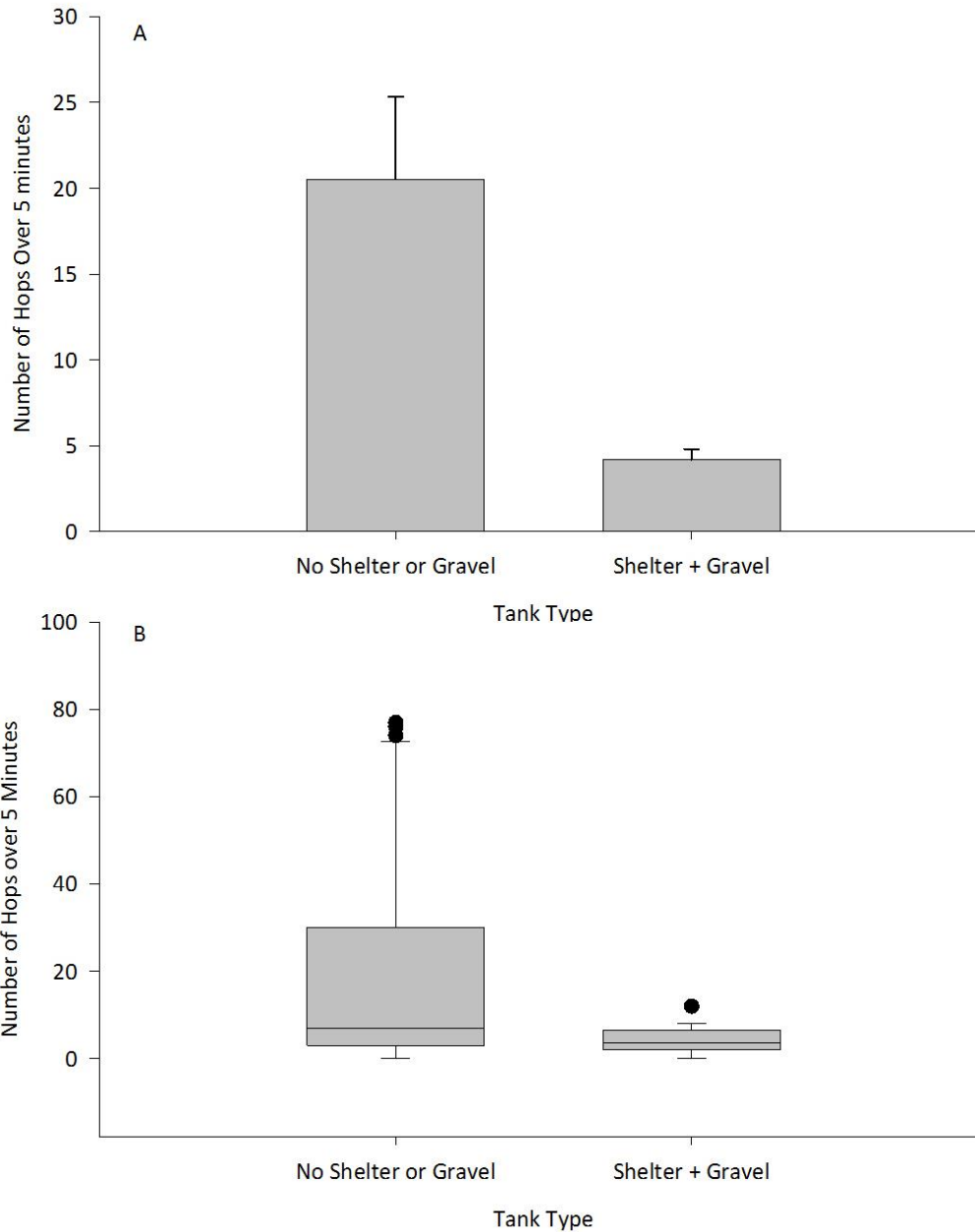
**Figure 2.5** Average hopping frequency (±SEM) in tanks with no shelter or gravel (n=31) compared to tanks equipped with a PVC shelter and gravel substrate (n=26) when 10mL of background water was delivered. Data expressed in a bar graph (A) to show averages, and a box plot (B) to show variance. Overall fish without shelter or gravel exhibit higher baseline hopping frequency than those with a shelter and gravel (p=0.036). It should be noted however that when no shelter or gravel are present, the variation in hopping frequency is much larger.
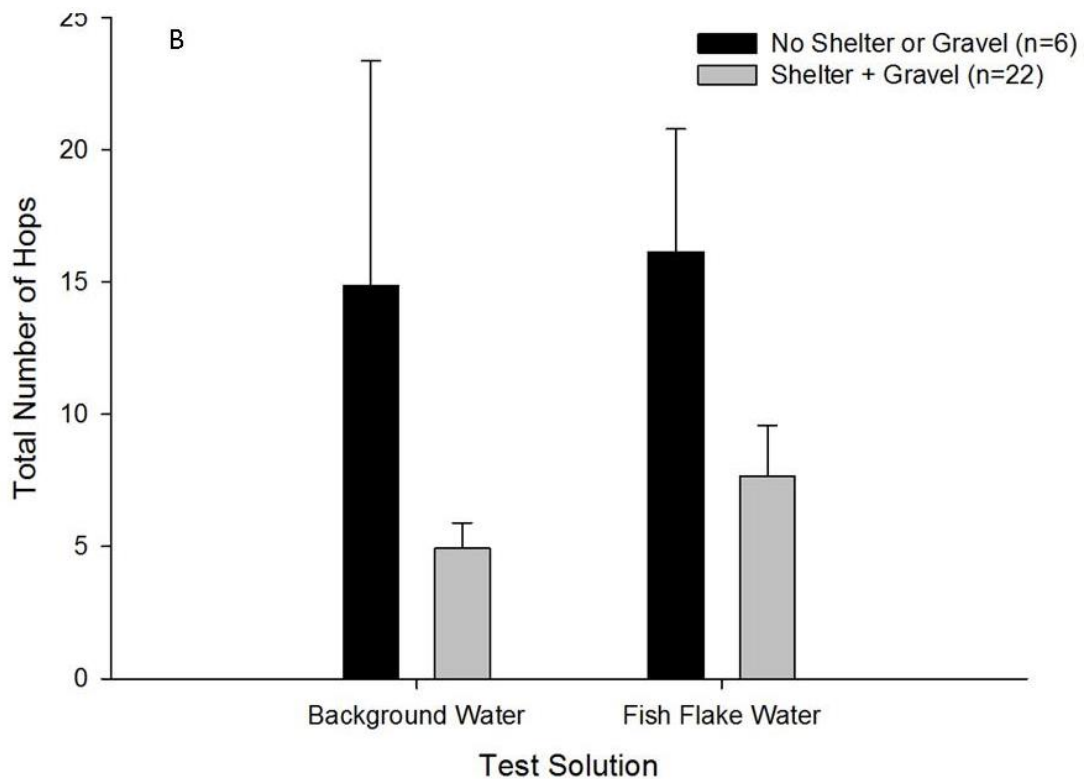
**Figure 2.6** Average number of hops (±SEM) performed by females over each 5 minute time period, an acclimation in which no test solution, only background water was delivered, and the stimulus period in which 10mL fish flake water was added to the tank under two different tank setups. The black bars indicate tanks that are absent of shelter or gravel, the grey bars indicate tanks with a PVC shelter and gravel substrate. When no shelter or gravel were provided, there was not a significant difference in hopping between the test solutions (p=0.44), but when a shelter and gravel were present, there was trend toward increased hopping frequency when exposed to fish flake water (p=0.07). Overall fish with no shelter/gravel hopped more than those without (p=0.07).
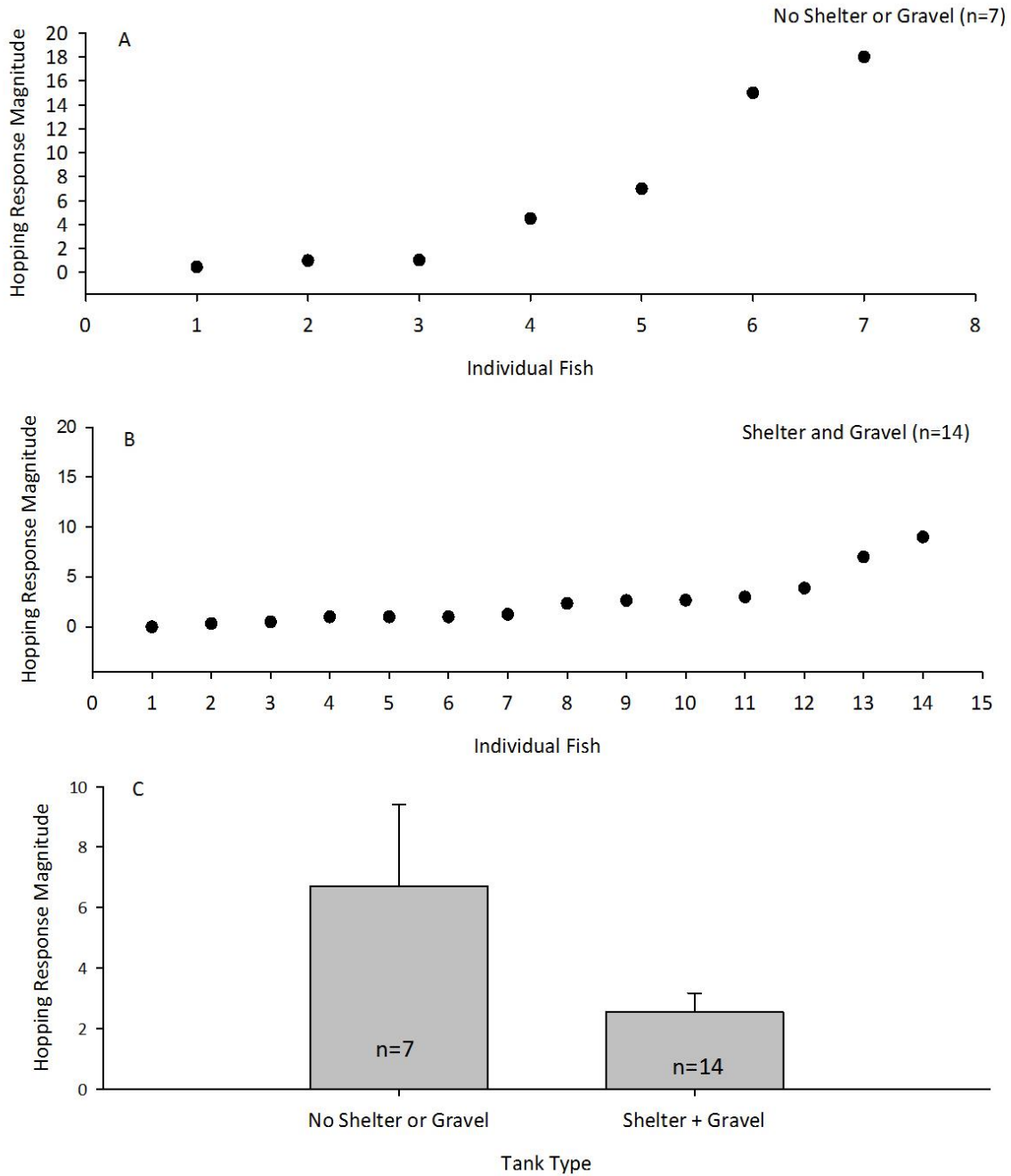
**Figure 2.7** Hopping response magnitude of individual fish in tanks without shelter and gravel (A) and thanks with a PVC shelter and gravel substrate (B) (data sorted by y-axis values) and average (±SEM) response magnitude within each tank type (C). Response magnitude was calculated by dividing the total number of hops over 5 minutes during exposure to 10mL of fish flake water by the total number of hops when exposed to 10mL of background water. The change in hopping frequency in response to fish flake water did not differ significantly in either tank type (P=0.29).

## 4. DISCUSSION

4.1 THE USE OF TANK ENHANCEMENTS SUCH AS SHELTERS AND GRAVEL

From these experiments, testing the effects of tank enhancements, (shelter + gravel substrate) it can be seen that overall, female round gobies spend significantly more time in a shelter (when provided) than in any other area of the tank. In addition, fish provided with a shelter exhibit lower baseline activity levels (measured in hopping frequency). These results support those seen by Marentette and Corkum (2008) in which over 60% of males did not leave the shelter, and spent more than 90% of their time immobile. In the wild, round gobies seek out shelters, perhaps as a method of predator avoidance (Belanger et al., 2003). It is likely that when no shelter is provided, fish exhibit higher activity levels due to searching for refuge (Edel, 1975). Interestingly, when presented with fish flake conditioned water (a potential food odour), fish provided with a shelter showed a stronger increase in hopping frequency and time spent in the inflow zone than those without a shelter or gravel (although neither showed a highly significant difference from baseline). This suggests that fish provided with a shelter and gravel are more 'comfortable' and able to respond to olfactory cues. It is possible that fish without shelters are more concerned with finding refuge from predators and thus are less likely to respond to food odours.

It is also important to note the differences in variation in hopping activity between fish in the absence/presence of a shelter (Fig. 2.5). This trend was most likely due to the fact that a large number of fish in the tanks without shelter or gravel move

continuously, throughout the 5 minutes.  These fish typically swam to the surface

repeatedly along the glass sides of the tank.  Not a single fish provided with a shelter

and gravel substrate ever hopped continuously, and rarely did they swim to the surface.

One explanation for this is that when no shelter is present, fish will spend their time

searching for a shelter, which has been documented in silver eels (*Anguilla rostrata*)

(Edel, 1975).  This large variation in activity could also be due to differences in

'personality' types.  There is a lot of evidence supporting the idea of individual

differences in behavioural phenotypes, typically in terms of boldness (or shyness)

(reviewed by Sih et al., 2004).  Because different fish may respond to the same cue in

different (sometimes opposite) ways, it may be beneficial for future studies to

distinguish fish based on their particular behavioural phenotype (Shamchuk & Tierney,

2012).  It is possible that gobies that exhibit exploratory behaviours are generally bolder

than other fish with lower activity levels, and future studies should attempt to

categorize responses to positive controls.  It should be noted however, that behavioural

phenotypes can be context specific.  Pumpkinseed sunfish (*Lepomis gibbosus*), exhibit

individual differences under different contexts (predator vs. novel food odours) but

these differences are not conserved (fish that are bold in one scenario are not always

bold in another) (Coleman & Wilson, 1998).  Therefore, if using a positive control, it

should be one from the same category as the test solution (foraging, alarm, or

reproduction).  Hormonal state can contribute to individual differences in behaviours.

Nest-holding male round gobies do not feed during reproduction (Kornis et al., 2012)

and are thus unlikely to show behavioural responses to food odours.  It is possible that

reproductive females may be less concerned with feeding cues than non-reproductive females.

4.2 LIST OF RECOMMENDATIONSFOR FUTURE STUDIES

1. Tank type should be chosen based on odours to be tested and experimental design
   - o Y-maze for choice experiments
   - o Small tanks (5L) work well for high throughput experiments
   - o Larger tanks (<90L) could be useful in quantifying attraction
2. The flow rate used in the experiments should be in the order of 100mL/min as this is behaviourally relevant (similar to flow rates created by male fanning)
3. Tank enhancements can reduce variability in behaviour, I suggest that future studies provide shelters and gravel substrate although more studies are required to determine the effectiveness of each of the enhancements.
4. The behavioral metrics used should include time spent in the inflow zone and hopping (if smaller tank) and distance travelled if a larger flume/tank
5. The sample size should be determined a priori using a power analysis, but in general, a sample size greater than 10 should be used.  In addition, responses should be categorized based on a scale of boldness-shyness or by different behavioural phenotypes

**5. FUTURE DIRECTIONS & CONCLUSIONS**

The experimental design for each of the studies reviewed was highly variable, and it would be beneficial for future studies to devise a standardized method of examining round goby behaviour in response to pheromones.  I have provided a list of recommendations in an attempt to increase the success of future studies on round goby behavioural responses to olfactory cues. The most important considerations should be flow rate, tank enhancements, behavioural metrics and sample size.  In general a standardized method of testing round goby olfactory mediated behaviours should be designed in order to elucidate the key pheromone components using in reproductive signaling.  In order to design future studies, I recommend a thorough examination of round goby reproductive behaviour and spawning both in nature and in a laboratory setting.  Future designs should attempt to create simplified versions of the round goby's natural spawning environment in order to achieve realistic behavioural responses.

**CHAPTER III – FEMALE MOVEMENT RESPONSES TO ISOLATED STEROID CONJUGATES RELEASED BY MALE ROUND GOBIES (*NEOGOBIUS MELANOSTOMUS*) AND TO SYNTHETIC ANALOGS**

*The work presented in this chapter was joint research with Dr. Eric Clelland and Dr. Michelle Farwell (specifically the HPLC protocol and ELISAs used)

**1. INTRODUCTION**

1.1 ROUND GOBY INVASION

The round goby (*Neogobius melanostomus*), a small benthic fish, native to the Black and Caspian seas, is a recent invader to the Laurentian Great Lakes (Jude et al., 1992). It was first discovered in the St. Clair River in early 1990 (Jude et al., 1992) and by 1997 had spread to each of the five Great Lakes (Charlebois et al., 2001). The round goby's colonization success can be attributed to its broad range of habitat and food types as well as its high fecundity and aggressive nature (Charlebois et al., 1997; Corkum et al., 1998). The round goby currently poses a threat to a variety of native species. It has competitively displaced several species of indigenous benthic fish such as the mottled sculpin *(Cottus bairdii)* (Bergstrom and Mensinger, 2009; Dubs and Corkum, 1996; Janssen and Jude, 2001) and is an egg predator of important game and commercial fish species including small mouth bass (*Micropterus dolomieu*) (Jude et al., 1995; Steinhart et al., 2004a). The round gobies may also contribute to the biomagnification of contaminants as dreissenids, which make up a large portion of their diet (Lederer et al., 2008), are contaminated with PCBs, organochloride pesticides, chlorinated benzenes, and dioxins (Richman and Somers, 2005), and gobies have been

found to be an increasingly popular food source of piscivorous fish (Steinhart et al., 2004b; Johnson et al., 2005; Dietrich et al., 2006; Truemper et al., 2006).  It is likely that, if left unchecked, the round goby could drive many species locally extinct.

1.2 ROUND GOBY REPRODUCTION

Round gobies reproduce when water temperatures range from 9-26 °C, and spawn multiple times per season (MacInnis and Corkum, 2000).  Males find and guard nesting territories from which males attract multiple females to deposit their eggs (Charlebois et al., 1997).  Males provide the sole parental care by regular inspection and ventilation of eggs (Meunier et al., 2009).  Males are thought to attract females through the use of visual, auditory and olfactory cues (reviewed by Kornis et al., 2012).  Reproductive male (RM) round gobies will fan using pectoral and caudal fins before egg deposition which could be a mechanism of odour dispersion (Meunier et al., 2009; Wantola et al., 2013).  It is evident that males release compounds that are attractive to reproductive females (RF), as RFs spend more time near the odour source when exposed to RM conditioned water (CW) and RM CW elicits olfactory responses in RFs (Belanger et al., 2004; Gammon et al., 2005; Corkum et al., 2006; Kasurak et al., 2012).  These findings suggest that males release some compound into the environment which can be used to attract females to nest sites.

1.3 POPULATION MANAGEMENT- PHEROMONE TRAPPING

Currently there are limited population management strategies in place for the round goby, and populations have expanded into each of the Great Lakes and the

Mississippi River basin (Kornis et al., 2012). Current research on the goby mating

strategy suggests that the exploitation of reproductive pheromones may be effective in

managing round goby populations. Manipulation of natural chemical communication in

an attempt to manage populations includes, population size and distribution

assessment, mating and migration disruption, promoting success of sterilized fishes,

repelling (alarm pheromones),and trapping (reviewed by Sorensen and Stacey, 2004).

Pheromone trapping employs the use of natural or synthetic versions of aggregation

chemicals used by the species of interest. This method of population control is species

specific due to the nature of pheromones. In addition, because these chemicals are

naturally being released into the environment already, their use is less likely to have

negative consequences on the surrounding ecosystem. They also typically have half-

lives of about a day meaning that they do not persist for long periods of time (Sorensen

and Stacey, 2004). In species with high densities, pheromone trapping can be used to

decrease numbers such that recruitment becomes density dependent (Twohey et al.,

2003; Sorensen and Stacey, 2004). Pheromones can be used to facilitate trapping in

order to remove individuals or to collect animals for sterilization (Sorensen and Stacey,

2004; Bergstedt and Twohey, 2007). Sex pheromones have the potential to greatly

increase the success of trapping efforts as they could be used to target and

remove/sterilize reproductive males or females, directly affecting the reproductive

success of a population. Because of this, sex and aggregation pheromones such as bile

acids and gonadal steroids (Doving and Selset, 1980) are of particular interest in

population management. The use of pheromones as bait in traps is commonly seen in

insect pest species, particularly in Lepidoptera (reviewed by McNeil, 1991; and Witzgall et al., 2010).  Recently, sex pheromone population management strategies have been investigated for other invasive fish species such as the sea lamprey (*Petromyzon marinus*) (Johnson et al., 2009) and the common carp (*Cyprinus carpio)* (Sorensen and Stacey, 2004).

Research on the biosynthesis and response to reproductive pheromones in fishes is limited.  Most of the literature available on the subject pertains to sea lamprey, salmonids, goldfish (*Carrassius auratus*), and gobies (as reviewed by Sorensen and Stacey, 1999; Stacey and Sorensen, 2002). These species commonly use gonadal steroids and prostaglandins as pheromones (Sorensen and Stacey, 1999, Stacey and Sorensen, 2002).  Often a multitude of hormonal by-products are released into the environment, but not all of them are used as reproductive signals. Because of this, fish live within a 'soup' of potential olfactory signals.  In order for individuals to recognize conspecific signals, the specific ratio of pheromone constituents can be very important (reviewed by Sorensen et al., 1998).  When applying putative pheromones in a lab setting, the receiver response can be optimized by presenting pheromones in a similar ratio to that released naturally by that species (Löfstedt et al., 1981; Beevor et al., 1999; Martin et al., 2013).  This also supports the idea that identification of key pheromone components should be not be done through single component tests, but rather by testing different combinations in which one of the constituents has been removed(as seen in, Millar et al., 1990; Reddy and Guerrero, 2000; De Silva et al., 2013; Levi-Zada et al., 2013).   A particular component may be attractive, but only when present in conjunction with

another component, thus testing it alone will not elicit a response, leading to a faulty conclusion.

To develop large scale, cost-effective methods of pheromone synthesis for trapping, it is important to investigate the basic biological information essential in a species' pheromone system. Thus, any strategy using pheromone manipulation will only be as good as our knowledge of the system. Specifically we need a thorough understanding of the biosynthesis, release, and reaction to pheromones. The overall aim of my study is to further our understanding of each of these processes in the round goby.

## 1.4 PUTATIVE STEROIDAL PHEROMONES OF MALE ROUND GOBIES

To determine if pheromone trapping is a viable option for round gobies, it had to first be determined if pheromones are used to attract females to the nesting sites. It has been well documented that water conditioned by reproductive males is attractive to reproductive females (Belanger et al., 2004; Gammon et al., 2005, Corkum et al., 2006). The attractive compound(s) can be isolated through the use of octadecylsilane (C18) cartridges and these compound(s) elicit olfactory (Belanger et al., 2004) and behavioural (Kasurak et al., 2012) responses. The next step was to characterize the compound(s); this was done through the use of high performance liquid chromatography (HPLC) in conjunction with mass spectrophotometry and enzyme-linked immunosorbent assays (ELISAs) (Katare et al., 2011). This study revealed that males release a novel steroid: 3α-hydroxy-5β-androstane-11,17-dione (**11-O-ETIO**) as well as four derivatives of this compound, **11-O-ETIO-3-s**: 3α-hydroxy-5β-androstane-11,17-dione 3-sulfate, **11-O-**

**ETIO-17-s:** 3α-hydroxy-5β-androstane-11,17-dione 17-sulfate, **11-O-ETIO-3-g:** 3α-

hydroxy-5β-androstane-11,17-dione 3-glucosiduronate,**11-O-ETIO-17-g**: 3α-hydroxy-5β-

androstane-11,17-dione 17-glucosiduronate.  These steroids are released in the ratio of

8:5:1.5:4:1 (when injected with GnRHa) (Fig. 3.1) (Katare et al., 2011; Farwell, in press).

When synthetic analogs of both sulfated and unconjugated 11-O-ETIO were presented

to NF during EOG recordings, responses were seen to both the unconjugated and -3 s

steroids, but not towards 17-s (Laframboise and Zielinski, 2011).  In addition, females

showed the strongest olfactory responses when these steroids were delivered at a

concentration of 10nM, and that no response was seen at or below 0.1nM (Laframboise

and Zielinski, 2011). RFs showed significantly higher EOG responses to fractions that

corresponded to the elution positions of conjugated rather than free steroids (when

using HPLC to isolate steroids from CW extracts) (Belanger et al., 2004).  In addition,

reproductive phase females were shown to be attracted to pooled fractions containing

the  conjugates of 11-O-ETIO, and avoid fractions containing unconjugated, 11-O-ETIO,

both delivered at 10nM (Kereliuk, 2009).  The above evidence combined suggests that a

conjugate of 11-O-ETIO, particularly 11-O-ETIO-3-s (due to high concentration and EOG

responses), may be a good candidate for initial behavioural testing in an effort to

develop an effective pheromone trapping protocol.

1.5 OBJECTIVES

In the current study, movement responses of female round gobies to various

combinations of 11-O-ETIO and its derivatives at both low and high concentrations will

be tested in an effort to elucidate key pheromone components used in round goby mate

attraction.  In order to test this, I performed two experiments (Table 1.1).  The goal of

the first experiment tested movement responses to fractionated RM CW containing

separated 5β-androstane constituents and to reconstituted blends of these fractions.

This was tested using the 'removal' technique in which fractions containing four of the

five steroids were delivered in each test solution.  The goal of the second experiment

was to expand upon the results of the first experiment by changing i) the concentration

of test solutions (through the use of synthetic 11-O-ETIO and its conjugates in the

natural release ratio but at 1μM, and decreased tank volume) ii) the tank setup (through

the addition of gravel substrate and a PVC shelter) and iii) focus on the role that 11-O-

ETIO-3-s plays in mate attraction (by testing it alone, as well as removed from the

steroid blend).   Based on a previous EOG study (Laframboise and Zielinski, 2011), and

studies in insects (Reviewed by Carde et al., 1998), by increasing the concentration I

expect to see an increase in female response.  In addition to using synthetic steroids, I

also tested the effects of tank enhancements (gravel substrate and a PVC shelter) on

female responses as this mimics a more natural environment (for other studies

employing enhancements, Tavolga, 1956, Gammon et al., 2005; Malavasi et al., 2009,

see also Chapter II of this thesis).  Overall this study will be the first to test female round

goby movement responses to both natural and synthetic blends of steroids using the

component 'removal' method.  The results of this study will be crucial for the successful

development of a pheromone trapping strategy for the round goby.

**Table 3.1** Details of experiments testing female responses to putative male steroid

pheromones.  Experiment 1 investigated round goby responses to fractionated

conditioned water and Experiment 2 investigated responses to synthetic steroids by

gobies provided with a shelter and gravel substrate.

| Experiment 1 | Experiment 2 |
|---|---|
| • Conducted May-July, 2012<br>• No shelter<br>• No gravel substrate<br>• 4L of water in each tank<br>• 10 nM 11-O-ETIO in CW extract<br>• Tested fractionated CW extract containing 0.01 nM 11-O-ETIO and 11-O-ETIO derivatives<br>• Tested for time spent near the inflow and distance moved | • Conducted May-July, 2013<br>• PVC tube shelter<br>• Gravel substrate<br>• 2L of water in each tank<br>• 10 nM 11-O-ETIO in CW extract<br>• Tested 1 μM synthetic 11-O-ETIO and derivatives<br>• Tested for time near the inflow, time in shelter, number of hops, gill ventilation rate |

## 2. **MATERIALS AND METHODS**

2.1 EXPERIMENTAL ANIMALS

**Collection and Housing**

Round gobies were collected by angling and seining from the Detroit River (Windsor, ON) and Lake Erie (Erieau, ON) from May to October, 2011, 2012, and 2013. Fish were held in accordance with University of Windsor animal care guidelines, and experimental procedures conformed to the guidelines of the Canadian Council of Animal Care. Fish were housed in 205L, gravel-lined, aerated, flow-through tanks held at 18±1°C under a constant photoperiod of 16:8 (L:D). Fish were fed daily with Nutrafin fish flakes (Tetramin, Inc.).

**Reproductive Status**

Fish were sexed based on the appearance of their urogenital papilla; males have an elongated, triangular papilla, whereas females have a more broad, rounded papilla (Corkum et al., 1998). Reproductive status of males was also determined by the presence of secondary sexual characteristics: a dark body, presence of a thick slime coat, as well as enlarged cheeks, urogenital papilla, and fins (Corkum et al., 1998). Reproductive females were classified as such based on the presence of a distended belly and slight orange coloration in the urogenital papilla (Corkum et al., 1998; Kasurak et al., 2012). After each experiment, reproductive status was confirmed for each individual post-euthanization on the basis of its gonadosomatic index ($I_g$), defined as gonad weight (testes, seminal vesicles, and mesochiral gland for males, ovaries for females)/total

body weight X 100 (Strange, 1996).  Fish were classified as reproductive only if $I_g > 1.3$

(males) or $> 8.0$ (females) (Belanger et al., 2004; Katare et al., 2011).

2.2 PREPARATION OF TEST SOLUTIONS

**Collection of Conditioned Water from Reproductive Males**

Conditioned water was collected from reproductive males (36.43g±1.82, mean

±SEM) using methods similar to those of Katare et al., (2011). Reproductive males were

given three consecutive injections of salmon gonadotropin-releasing hormone analogue

(dissolved in 0.7% saline) (sGnRHa Syndell Labs, Vancouver, BC).  The first injection

volume was calculated as 0.5% body weight and then the male was placed in a one litre

tank of dechlorinated water.  After eight hours, this one litre of conditioned tank water

was collected and stored at 4°C, and each male was given a second injection (at a lower

dosage) of sGnRHa (1µg) and placed in another one litre tank with dechlorinated water.

After 16 hours, the one litre of conditioned tank water was collected and again stored at

4°C.  Each male was given a third and final injection of sGnRHa (again of 1µg) and placed

in a one litre tank with dechlorinated water. After eight hours, the one litre of

conditioned tank water was collected and stored at 4°C for a maximum of 24 hours.

Males were then euthanized and reproductive status confirmed by GSI.

**Preparation of Conditioned Water Extract**

Reproductive male CW contains steroids shown to be attractive to female round

gobies (Tierney et al., 2012; Gammon et al., 2005).  These steroids can be extracted

from conditioned water by passing each one litre sample through activated Sep-pak C18

cartridges (Part #: WAT020515 Waters, Milford, MA), washing the cartridge with water,

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and eluting with 5 mL of methanol (Fig. 3.2) (as per Katare et al., 2011). All methanol

extracts were pooled annually (2011: n=96 L/31 males), 2013: n=21 L/7 males). Pooled

samples were dried using a vacuum concentrator (Labconco, Kansas City, MO) and

resuspended in MeOH at 10 times concentration for storage at -20°C (i.e. if originally

5mL CW extract, this was dried and resuspended in 500µL for storage) (Fig. 3.2). For

Experiment 1 (performed in 2012), 'CW extract' test solution was prepared by diluting

5µl of 10X concentrated stock CW extract (from 2011) into 10mL of dechlorinated

water. In doing this, I attempted to replicate the concentrations of steroids found in the

original 1L of CW (5mL extract from 1L) (Fig. 3.2). ELISAs were used to verify the

immunoreactivity of 11-O-ETIO in this CW test solution was at 10nM (same

concentration used by Tierney et al., 2013) For Experiment 2 (performed 2013), 'CW

extract' test solution was prepared by diluting 173 µl of 10X concentrated stock CW

extract (from 2013) into 10mL of dechlorinated water. An ELISA determined that the

concentration of 11-O-ETIO in test solutions delivered was ~10nM.

**HPLC Fractionation of 11-O-ETIO Derivatives in Conditioned Water Extract (Experiment 1)\***

High performance liquid chromatography (HPLC) was used to separate steroids

in the conditioned water extract. A Nova-Pak®HR C18 6µm 60Å prep column (Waters,

Milford, MA) was used in conjunction with an acetonitrile (ACN) -water gradient

containing 0.025% trifluoroacetic acid (TFA) as the mobile phase (Waters, Milford, MA).

A linear gradient was applied from 11% ACN-0.025% TFA: 89% water-0.025% TFA

(buffer A) to 89% ACN-0.025% TFA: 11% water-0.025% TFA (buffer B) over 50 minutes.

A 100μl sample of 10X CW extract was dried and dissolved into 400.5μl buffer A and

49.5μl buffer B.  This 450μl was then injected into the column and the flow rate was

1mL min$^{-1}$. Fractions were collected at 1-min intervals and dried in a vacuum centrifuge

at 35°C, and the dried residues were later dissolved in 100μl of MeOH in 1 mL glass vials

and stored at -20°C. ELISAs were employed in conjunction with solvolysis and

glucosiduronidase application to each 100μl  fraction to determine the elution times and

amounts of each of the steroids by a RM in 1L of water: 11-O-ETIO-17-s (fractions 17

and 18, 34401.65 pg/mL), 11-O-ETIO-3-s (fractions 21 and 22, 86032.21 pg/mL), 11-O-

ETIO-17-g (fractions 24,25, and 26, 38571.07pg/mL), 11-O-ETIO-3-g (fractions 27 to 31,

134846.30 pg/mL) and 11-O-ETIO (85855.70pg/mL) (Fig. 3.1) (Farwell et al., in press).

For Experiment 1, 27μl of each individual fraction (in MeOH stock) was diluted into 50

mL of dechlorinated water.  Analysis of the test solutions determined that the immuno-

reactivity of 11-O-ETIO was 0.1nM.  It should be noted that analysis of these test

solutions was performed post-hoc and it was discovered that these concentrations were

lower than ideal for olfactory sensory threshold for the round goby (Laframboise and

Zielinski, 2011) likely due to isolation inefficiencies.  Test solutions for Experiment 1

were created by pooling each of the fractions of interest (Fig. 3.1) into 10mL of

dechlorinated water.  Test solutions differed by the exclusion of one of the five steroids.

This method of subtracting steroids rather than applying individual steroids was used in

order to factor in the possibility that multiple steroids are required to elicit a response

(Millar et al., 1990; Reddy and Guerrero, 2000; De Silva et al., 2013; Levi-Zada et al.,

2013).  The test solutions used were as follows (see also Fig. 3.1.1, Chapter I of this

thesis):

1. Conditioned water extract (CW ex) derived from 2011 RM contained 10 nM 11-
   oxo-ETIO.

2. 'Minus 17s': Fractions 19-31 containing steroids in the 0.1 nM range (11-O-ETIO-
   3-s, 11-O-ETIO-17-g, 11-O-ETIO-3-g, and 11-O-ETIO)

3. 'Minus 3s': Fractions 16-21 and 24-31 (11-O-ETIO-17-s, 11-O-ETIO-17-g, 11-O-
   ETIO-3-g, and 11-O-ETIO)

4. 'Minus 17g and 3g': Fractions 16-24 and 27-31 (11-O-ETIO-17-s, 11-O-ETIO-3-s,
   and 11-O-ETIO)

5. 'Minus Free': Fractions 16-26 (11-O-ETIO-17-s, 11-O-ETIO-3-s, 11-O-ETIO-17-g,
   and 11-O-ETIO-3g)

6. Vehicle Blank: 10mL dechlorinated water

**Preparation of Synthetic Steroid Solutions (Experiment 2)**

All synthetic steroids were obtained from Steraloids, Inc. (Newport, RI, USA).

These included, (note that 11-O-ETIO-17-g is not commercially available):

- 11-O-ETIO (CAS #739-27-5: 5β-ANDROSTAN-3α-OL-11, 17-DIONE)
- 11-O-ETIO-3-s (Catalogue ID A3500-000: 5β-ANDROSTAN-3α-OL-11, 17-DIONE,
  SULPHATE, SODIUM SALT)
- 11-O-ETIO-17-S (Catalogue ID A3232-000: 5\-ANDROSTAN-3|, 17\-DIOL-11-ONE-
  17-SULPHATE, SODIUM SALT)
- 11-O-ETIO-3-g (CAS #17181-16-7: 5β-ANDROSTAN-3α-OL-11, 17-DIONE
  GLUCOSIDURONATE)

Each was dissolved in methanol (in 10µg/mL and 1mg/mL MeOH stocks stored at -20°C). All test solutions for the behavioural trials were prepared by drying and resuspending in 10mL of dechlorinated tap water to desired concentrations (see below). Original steroid ratio was maintained such that 11-O-ETIO-3-s was at 1µM (see Katare et al., 2011) because the ratio of pheromone constituents can play a large role in response rates of receivers (Millar et al., 1990; Reddy and Guerrero, 2000; Poling et al., 2001; De Silva et al., 2013; Levi-Zada et al., 2013).

2.3 BEHAVIOURAL ASSAY AND ANALYSIS

**Experiment Set Up**

Experiments used four litre tanks (8cm X15 cm X15 cm). Tanks were under constant flow through (130mLmin$^{-1}$) of dechlorinated water (18°C ± 2). Four tanks were used simultaneously, each visually isolated from one another by wrapping the tanks in black plastic. Each of the tanks was visually divided into 8 sections ('boxes') by placing a grid under the glass-bottomed tank (Fig. 3.3). Experiments took place between May to September, in the afternoon, and under a low-light setting (one florescent bulb). In addition to inflow of background water, each tank was equipped with a 1mL glass pipette through which the test odours and background water were delivered. On the day prior to the experiment, four females were transferred from housing tanks and placed individually into each of the four experimental tanks. A cooler containing dechlorinated (background) water provided constant flow through the glass pipette via a peristaltic pump. Test solutions were delivered to the tank by turning a valve, thus

changing the source from the cooler to a 30mL plastic syringe containing the test

solution (10mL of test solution, flows for approximately 1.5 minutes). After the solution

was added, the valve was switched back to continuous flow of dechlorinated water.

Behaviours were recorded in 30 s time bins (Tierney et al., 2013). After experiments,

fish were euthanized and GSI was recorded.

**Flow Analysis**

Dye and steroid tests were used to verify the change in concentration of test

solutions over time (Fig. 3.4). Dye tests used 5% methylene blue to illustrate the time it

took the test solution to reach the tank, the outflow, and wash out. Synthetic 11-O-ETIO

(at 1μM) was used in conjunction with an ELISA to test specific concentration changes. I

found that test solutions delivered existed in a concentration gradient from 90-210s

post-delivery. The solution became evenly diluted (by two orders of magnitude) by five

minutes post-delivery. In Experiment 1, the dye had cleared the tank by 20 minutes

post-delivery, and 40 minutes for the Experiment 2 (Fig. 3.4B).

**Experiment 1**

All females (n=30, mean TL±SE 8.10±0.54) were placed in the experimental tanks

(Fig. 3.3A) in the morning for three hours prior to the administration of the test

solutions in order to acclimate the fish to the new environment. Experiments took place

between 12:00-4:00pm. Fish were exposed to six consecutive, ten minute treatments

(in randomized order) each preceded by a fifteen min acclimation period, and followed

by a ten minute recovery period (dye tests show that the test solutions cleared the tank

by ten minutes post-delivery) (Fig. 3.5). The next treatment began immediately following the 10 minute recovery period. For this experiment, fractions 16-31 were used, and depending on the treatment, a particular set of fractions was removed (Fig 1). Again, test solutions delivered were as follows: CW ex, 'Minus 17s,' 'Minus 3s,' 'Minus 17g and 3g,' 'Minus Free,' and vehicle blank. The immunoreactivity of 11-O-ETIO in CW extract was 10nM, and in the 'minus' test solutions was 0.1nM.

Fish behaviour and location in the tank was recorded using CCD cameras (Matco, St. Laurent, QE) and analyzed using Ethovision recording software (blind) (EthoVision®XT 7, Noldus, Leesburg, VA). On occasion, Ethovision failed to correctly track fish leading to unequal sample sizes across treatments. For analysis, the last 5 minutes of the fifteen minute 'acclimation period,' the first 5 minutes of the 'stimulus period' and last 5 minutes of the 'recovery period' were used (Fig. 3.5A). For each fish, percent time in the inflow zone and distance moved (measured in 'number of boxes') were measured.

**Experiment 2**

These experiments utilized the same rectangular glass aquaria, but the volume was dropped to 2.8L of dechlorinated water in order to decrease the amount of test solution required to reach detectable levels (Laframboise and Zielinski, 2011). These tanks remained on continuous flow through of aerated dechlorinated water. Tanks were equipped with half of a secured, white, two and a half inch long piece of PVC tubing (2 in length, 1 in diameter) and a layer of white/grey gravel substrate (Fig. 3.3B). In pilot studies, as well as in Experiment 1, approximately 50% of fish did not move throughout the entire experiment. In an effort to increase the number of fish

responding, these tank enhancements were added in an attempt to increase the

number of fish responding (see Murphy et al., 2001 for similar design). Females (n=30,

mean TL±SE 9.50±0.48), were placed in experimental tanks (one female per tank) at

4:00pm on the day prior to the experiment to provide longer acclimation times (than

Experiment 1) again in an effort to increase response rates. The order of delivery of

each test solution was randomized on the day of the experiment. Each test consisted of

four test solution delivery periods. Each delivery period was made up of three time

periods: acclimation, stimulus and recovery (Fig. 3.5B). During the ten minute

acclimation period, no solution was added and behaviour was recorded for the last five

minutes. The test solution was then added. The stimulus period (the time in which the

solution exists in a concentration gradient) occurs between 1 minute- 3minutes post-

delivery (Fig. 3.4B). The test solution became evenly mixed by five minutes post odour

delivery (dilutes by two orders of magnitude). After each test, fish were given a thirty

minute recovery period. During this time, the test solution diluted, and was cleared

from the tank (clearing period is extended from Experiment 1 due to higher

concentration of test solution) (Fig. 3.4B). The next trial period began immediately

following this thirty minute recovery. In this experiment, females were exposed to

blends of synthetic steroids delivered in the respective ratio observed in CW with 11-O-

ETIO-3-s at a concentration of 1μM. Methanol stocks of synthetic steroids were dried

and resuspended in 10mL of dechlorinated water (per fish). It should be noted that in

this experiment, all MeOH was removed from the samples, whereas in the previous

experiment, the MeOH was not removed. This should not greatly affect the experiment

as the levels of MeOH in the previous experiment were very low (at most 100µL in the

entire 4L tank).  The reason this change was made was to avoid any possible effects of

MeOH on female responses as gobies do show olfactory responses to MeOH when using

EOGs (Dr. Allyson Laframboise, pers. comm.).

Test solutions included;

- CW extract: from 2013 RMs (contained $10^{-8}$M 11-O-ETIO concentration)

- 3s Alone: 17µl of 1mg/mL synthetic 11-O-ETIO-3-s

- Minus 3s: Synthetic 11-O-ETIO-17-s (9µl), 11-O-ETIO-3-g (28µl), 11-O-ETIO (50µl)
  at 1mg/mL

- All Steroids: Synthetic 11-O-ETIO-17-s (9µl), 11-O-ETIO-3-s (17µl), 11-O-ETIO-3-g
  (28µl), 11-O-ETIO (50µl) at 1mg/mL

It should be noted that I tested the synthetic 11-O-ETIO-3-g even though the

preparation is a racemic mixture (M. Revington, U Windsor, pers. Comm.).  Both S and

R-enantiomers exist which could have an effect on olfactory reception (Hobson et al,

1993).

**Behavioural Analysis: Experiment 2**

Fish were observed from a side view of the tank, and movements were recorded

directly using a voice recording device.  In addition, fish activity was recorded from

above using the same CCD cameras as in 2012.  In order to quantify responses to test

solutions, fish activity was recorded during the last five minutes of the acclimation

period  and for five minutes post odour delivery (0:30s-5:30 minutes post-delivery).  In this experiment, trials could only take place one fish at a time (due to directly observing behaviours rather than video recording) so in order to complete four trials within the time constraints, activity in the recovery period was not recorded.  Fish movements were quantified in thirty second time bins.  Activity recorded included: hops (movements less than one body length) (see Murphy, 1999), time spent in the inflow zone (25% of tank closest to test solution source), time spent in the PVC shelter, and gill ventilation rate.

2.4 DATA ANALYSIS AND STATISTICAL METHODS

All statistical analyses were performed using Sigmaplot®11.0 (Systat Software, Inc., San Jose, CA).

**Time Spent in the Inflow Zone (Experiment 1 and 2) or in PVC Shelter (Experiment 2)**

Attraction can be quantified by the amount of time spent near an odour source (Gammon, 2005).  In Experiment 1, this was measured using the Ethovision software which detects the fish's location within the tank ten times per second.  If the fish was in the inflow zone (25% of the tank closest to the odour source) the software reported a value of one, if in any other location it reported a zero.  For Experiment 2, the amount of time (in seconds) spent in the inflow zone was recorded directly from watching the video recordings.  The percent of time the fish spent in the inflow zone per minute was calculated.  The change of time spent in the inflow zone was also compared to GSI using a linear regression, in order to further investigate the relationship between reproductive

status and attraction to RM CW (this was not done for Experiment 2 due to small sample size). The change in the percent time spent in the inflow zone was calculated by subtracting the average percent time (per minute) each fish spent in the inflow zone over the last five minutes of the acclimation period from the average percent time during the first five minutes of the stimulus period. Because round gobies are sessile, females that remained in the inflow zone for greater than 60% of the acclimation were excluded from the analysis (Tierney et al., 2012). A Friedman repeated measures analysis of variance on ranks was used to test for statistical differences across each of the time periods (within fish of similar reproductive state and within a test solution). A Mann-Whitney rank sum test was used to test for differences in percent time spent in the inflow zone during the stimulus periods between RFs and NFs (within test solutions). A Kruskal-Wallis one way analysis of variance on ranks with a Holm-Sidak post hoc test was used to test for differences within fish of a reproductive state, across the stimulus periods of each of the test solutions (note, RF data was normalized using an arc sin transformation). The change in percent time in the inflow zone between the acclimation and following stimulus period was calculated by subtracting the average percent time in the inflow zone per minute during acclimation from that of the stimulus period. A Mann-Whitney non-parametric analysis was used to test for differences between RFs and NFs, and a Kruskal-Wallis, non-parametric one-way analysis of variance was used to test for differences across test solutions.

For Experiment 2, Time spent in the PVC shelter was analyzed using a one-way repeated measures ANOVA to compare across test solutions (with fish of the same

reproductive status) and a Mann-Whitney rank sum test to test for differences between RFs and NFs within each test solution.

**Distance Travelled (Experiment 1)**

Experimental tanks were visually divided into 8 'boxes' (Fig. 3.4A).  For Experiment 1, distance travelled was measured using the number of boxes each fish travelled over each 30 second time bin (see Tierney et al., 2013).  Change in distance travelled was calculated by subtracting the total number of boxes travelled by each fish during the last five minutes of the acclimation period from the first five minutes of the stimulus period. A linear regression was used to compare the change in distance travelled to GSI.  A Mann-Whitney non-parametric analysis was used to compare the change in distance travelled between RFs and NFs for each test solution.  A Kruskal-Wallis non-parametric one-way analysis with Dunn's difference of ranks was used to compare averages across test solutions (within a reproductive state).  Distance travelled was not analyzed in Experiment 2 as hopping was used to quantify movement instead.

**Hopping Behaviour (Experiment 2)**

For this study, a hop was defined as a short forward or vertical movement, less than one body length.   Because round gobies typically move about the aquaria using this hopping motion, particularly in response to food (pers. obs.), an increase in frequency may indicate searching behaviour (Murphy, 1999; Kereliuk, 2009).  The number of hops was tallied every 30 seconds.  The number of hops during the acclimation period was compared to the number during the stimulus period using a

paired t-test to compare fish of the same reproductive status across test solutions, and a Mann-Whitney rank sum test to compare between RFs and NFs within each test solution. A one-way repeated measures ANOVA with a Holm-Sidak post hoc test was used to test for statistical differences in the number of hops performed during only the stimulus period across test solutions (within fish of the same reproductive status). Change in hopping activity was calculated by subtracting the total number of hops performed by each fish during the last five minutes of the acclimation periods from the first five minutes of the stimulus period. A Friedman repeated measures analysis of variance on ranks was used to test for differences in hopping behaviour across test solutions. I also looked at the response magnitude of hopping behaviour. This was calculated by dividing the number of hops performed during the entire five minutes of the stimulus period by the entire five minutes of the acclimation period. Statistical tests were not run on this data as it was meant to visually demonstrate the trends in this behavioural metric.

**Gill Ventilation (Experiment 2)**

Gill ventilation rate (number of opercular openings per minute) is commonly used as a metric for round goby olfaction (Murphy et al., 2001; Belanger et al., 2004; Tierney et al., 2012). In some benthic fish, ventilation rate is positively correlated with water flow through the naris suggesting that gill ventilation is correlated with olfaction (Nevitt, 1991). Average gill ventilation rates were measured directly from video footage and during the experiment. The number of gill ventilations over ten seconds was

measured once during each 30 second time bin of the acclimation and stimulus periods.

The change in gill ventilation rate was calculated by subtracting the average gill

ventilation rate during the acclimation period from the average gill ventilation rate

during the stimulus period. Student's t-tests were used to test for differences between

RF and NF within each test solution and a one-way repeated measures ANOVA was used

to test for differences in gill ventilation rates across test solutions.  The gill ventilation

rate during the last 5 minutes of the acclimation period was compared to the rate

during the first 5 minutes of the stimulus period using a paired t-test.
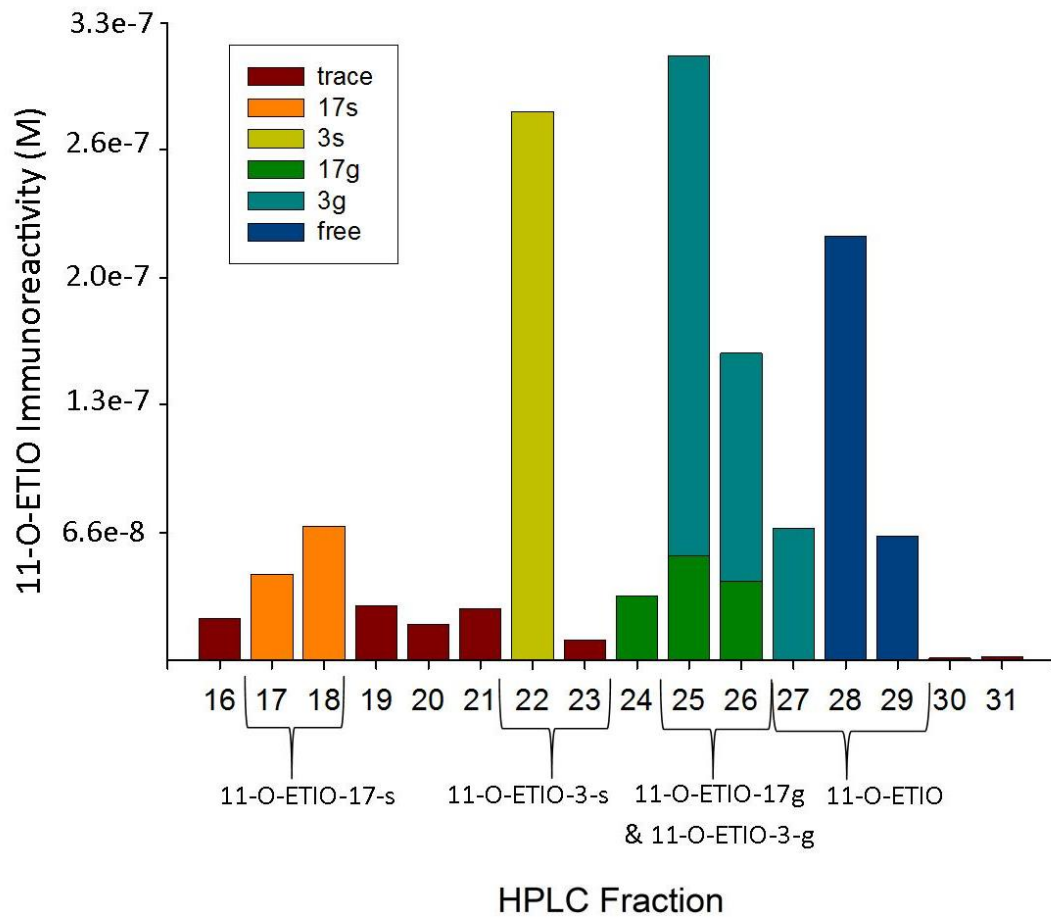
**Figure 3.1.** Concentration of 11-O-ETIO and its conjugates within 1L of reproductive male conditioned water. The y-axis denotes the immunoreactivity of 11-O-ETIO (M), the x-axis denotes the HPLC elution times for each of the steroids (in minutes).
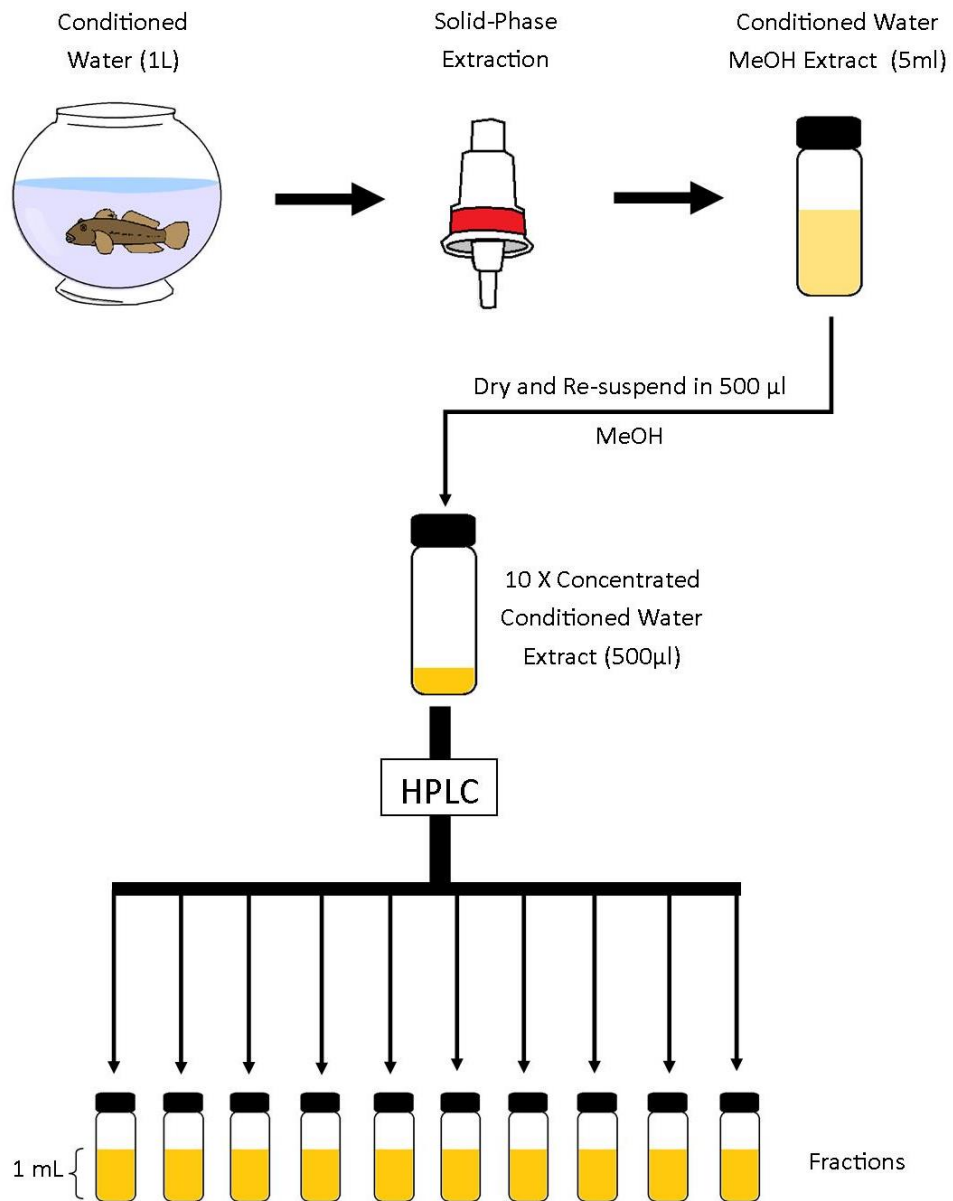
**Figure 3.2** Isolation process of steroids released by reproductive males from 1L of conditioned water to fractionation via HPLC. Each 1mL fraction contains the amount of steroids released by 1 reproductive male in 1L of water over an 8-16hour period.

**A: Experiment 1**



**B: Experiment 2**



**Figure 3.3** Experimental tank set up for Experiment 1(A) and Experiment 2 (B). Experiment 1 tanks were void of any tank enhancements (no shelter or gravel substrate), whereas Experiment 2 tanks were equipped with gravel substrate and a PVC shelter.   The shaded box on the left represents the 25% of the tank closest to the test solution source.
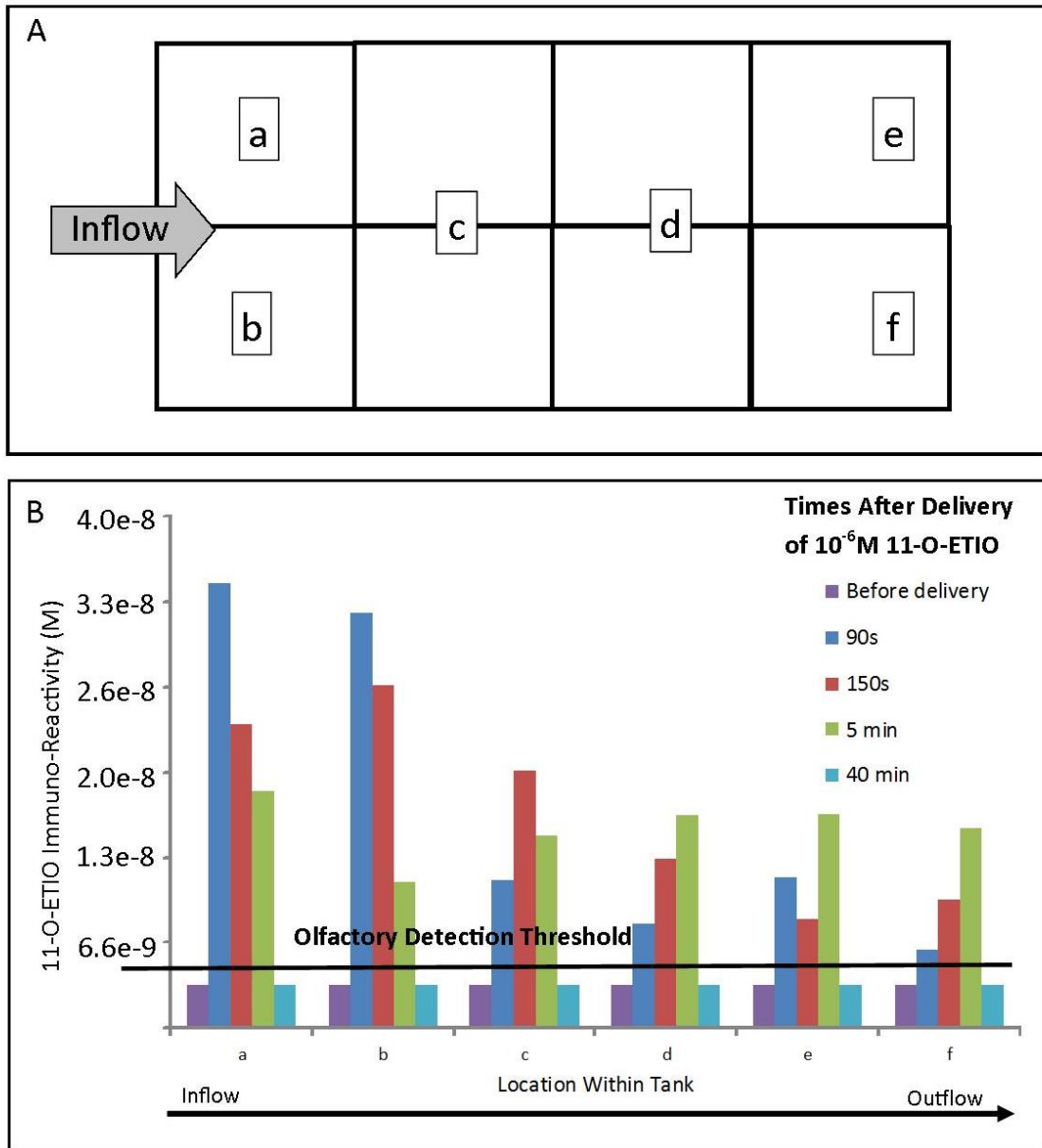
**Figure 3.4** Diagram depicting tank 'box' grid and solution inflow (A). 10mL of 1µM 11-O-ETIO was delivered to the tank and samples were taken at specific locations (lower case letters) within the tank over time. Concentration analysis via ELISA (B) depicts the dilution of 11-O-ETIO within the Experiment 2 test tanks before delivery, at 1:30, 2:30, 5 and 40 minutes post-delivery (B). The test solutions exist in a concentration gradient from time 1:30-2:30 and are evenly mixed by 5 minutes post-delivery. The black line signifies the olfactory threshold for 11-O-ETIO in round gobies (Laframboise & Zielinski, 2011).

A

Experiment 1: 35 minutes each
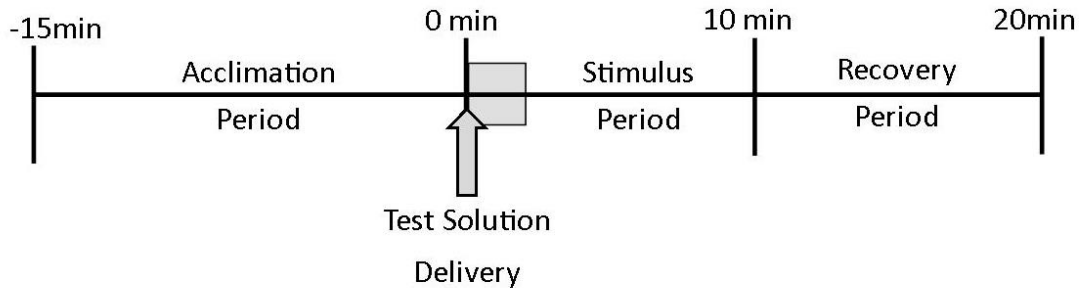


B

Experiment 2: 50 minutes each
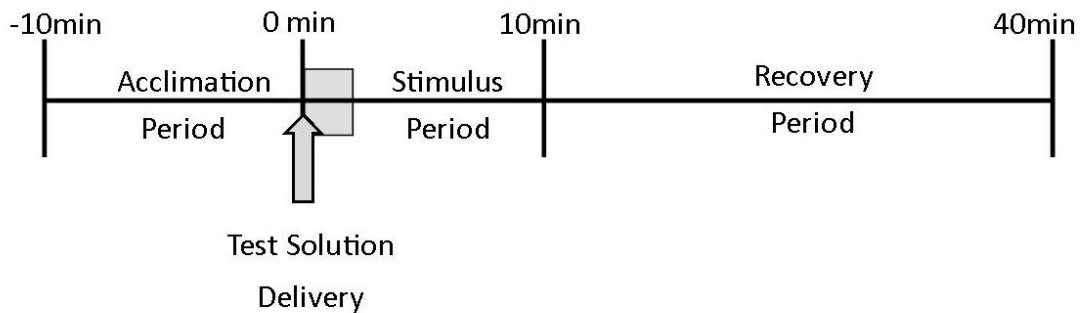


**Figure 3.5** Diagram describing the time periods for Experiment 1(A) and 2(B). Each experiment is broken up into an acclimation period (only background water is delivered), a stimulus period in which test solution is delivered for 90 seconds (light gray box) and exists in a concentration gradient, and a recovery period during the washout of test solution. Experiment 1 tests occurred over a 35 minute period whereas Experiment 2 tests occurred over a 50 minute period due to longer washout times of high concentration test solutions.

**3. RESULTS**

In order to provide a detailed and thorough examination of the data collected, often both the individual responses as well as the means are expressed in the figures.

3.1 EXPERIMENT 1: ACTIVITY IN UNFRACTIONATED AND FRACTIONATED CONDITIONED WATER EXTRACT

**Time Spent in the Inflow Zone**

*Unfractionated CW Extract*

When unfractionated CW extract containing 10 nM 11-O-ETIO flowed into the tanks, some NF (n=29) and RF (n= 22) did not change time in the inflow zone, some decreased time in the inflow zone, and others increased time in the inflow zone. Overall, there was no significant difference in time spent in the inflow zone during the 5 minute stimulus period between RF (n=22) and NF (n=29) (U=317.00, P=0.98, Fig. 3.6A and B). When comparing time spent in the inflow zone across the three time periods (acclimation, stimulus, and recovery) (5 min each) it was found that neither RF nor NF spent significantly more time in the inflow zone during any of the 5 min time periods (RF: $\chi^2$=0.70, P=0.70, NF: $\chi^2$=2.14, P=0.34, Fig. 3.6A and B). The change in time spent in the inflow zone was compared to GSI using a linear regression (n=51). The change in time spent was not correlated to GSI ($r^2$= 0.01, P=0.54) (Fig. 3.7).

*Fractionated Conditioned Water Extract*

Time in the inflow zone during the introduction of combined fractions containing a subset of the released steroids was investigated. I expected more time spent in the inflow zone during the introduction of fractions that contained attractive

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pheromones, and no change in the time spent in the inflow zone during the introduction

of preparations that lacked these pheromones, or that contained pheromone levels

below the threshold for a behavioural response. Since the inflow zone occupied 25% of

the tank area, if a solution was not attractive fish were expected to spend 25% of their

time in the inflow zone. When the vehicle blank (n=7) was introduced into the tank, 4

individuals spent more time in the inflow zone (Fig. 3.8). When comparing the time

spent in the inflow zone in response to the vehicle blank, fish (NFs) moved significantly

more during both the stimulus period and recovery period (F=14.85, P=0.02) (Fig. 3.9).

RFs (n=10) and NFs (n=19) were exposed to combined fractions 16 and 19-31,

containing approximately 0.1 nM 11-O-ETIO, 11-O-ETIO-3-s, 11-O-ETIO-17-g, and 11-O-

ETIO-3-g (but without isolated 11-O-ETIO-17-s in fractions 17 and 18). When individual

responses were viewed, all RFs showed increased time in the inflow zone, and most NF

spent more time in the inflow zone during the delivery of the test solution than prior to

test delivery (Fig. 3.8). However, there was no significant difference in time spent in the

inflow zone during the stimulus period between fish of either reproductive status

(U=93.00, P=0.94, Fig. 3.8). A comparison of time spent in the inflow zone across time

periods (acclimation, stimulus, and recovery) did not reveal any significant differences

for RFs ($\chi^2$=1.83, P=0.57) or NFs ($\chi^2$=0.16, P=0.92) although it does appear as though

some of the fish did increase their time spent in the inflow zone within the first minute

of delivery and then this response returned to baseline levels (Fig. 3.10).

RFs (n=10) and NFs (n=10) were exposed to combined fractions 16-21 and 24-31

(containing 0.1 nM 11-O-ETIO, 11-O-ETIO-17-s, 11-O-ETIO-17-g, and 11-O-ETIO-3-g, but

lacking 11-O-ETIO-3s, fractions 22 and 23). Neither RFs nor NFs significantly changed

the percent of time in the inflow zone during this treatment (Fig. 3.8). There was no

significant difference in time spent in the inflow zone during the first 5 minutes of the

stimulus period between fish of either reproductive status (U=42.50, P=0.86, Fig. 3.11).

A comparison of time spent in the inflow zone across each of the 5 min time periods

(acclimation, stimulus, and recovery) did not reveal any significant differences for RFs ($\chi^2$=0.72, P=0.81) or NFs ($\chi^2$=1.91, P=0.53) (Fig. 3.11).

RFs (n=11) and NFs (n=17) were exposed to combined fractions 16-24 and 27-31

containing; 0.1 nM 11-O-ETIO, 11-O-ETIO-17-s, and 11-O-ETIO-3-s), but lacking fractions

25 and 26 (11-O-ETIO-17-g and 11-O-ETIO-3-g). The majority of NFs and RFs spent more

time in the inflow zone during this treatment (Fig. 3.8). However, there was no

significant difference in time spent in the inflow zone during the first 5 minutes of the

stimulus period between fish of either reproductive status (U=71.50, P=0.29, Fig. 3.12).

A comparison of time spent in the inflow zone across time periods (acclimation,

stimulus, and recovery) did not reveal any significant differences for RFs ($\chi^2$=3.71,

P=0.16) or NFs ($\chi^2$=4.96, P=0.08) (Fig. 3.12).

RFs (n=11) and NFs (n=10) were exposed to a blend containing fractions 16-26

(0.1 nM 11-O-ETIO-17-s, 11-O-ETIO-3-s, 11-O-ETIO-17-g, and 11-O-ETIO-3-g), but not to

free 11-O-ETIO (fractions 27-29). About half of the NFs spent more time and half spent

less time in the inflow zone during this treatment (Fig. 3.8). There was no significant

difference in time spent in the inflow zone during the stimulus period between fish of

either reproductive status (U=88.00, P=0.10, Fig. 3.13). However, a comparison of time

spent in the inflow zone during the recovery period revealed that RFs spent significantly more time in the inflow zone than during the acclimation and stimulus periods (t=2.62, P=0.02), whereas NFs did not spend significantly more time in the inflow zone during any of the time periods (F= 0.44 P=0.65) (Fig. 3.12). These findings suggest that RF exhibited a delayed attraction to fractions 16-26, which contained 11-O-ETIO derivatives, but not the free 11-O-ETIO.

*Summary of Experiment 1a: Time Spent in Inflow Zone.*

I expected to see more fish with increased time at the inflow zone when pheromones were introduced into the tank, and fewer fish with more time at the inflow zone when pheromones were absent from the test solution.  Overall, the fish did not spend more time in the inflow zone when CW extract was introduced.  The majority of the test fish increased time at the inflow zone when preparations containing 11-O-ETIO-3-s and 11-O-ETIO, but missing fractions containing 11-oxo-ETIO-17-s or the glucuronated 11-O-ETIO were introduced into the tank. When fractions containing 11-O-ETIO-3-s (fractions 23 and 24) were left out of the test solution, none of the RFs or NFs tested significantly changed their time spent in the inflow zone.  When free 11-O-ETIO was removed from the test solution, most RFs and NFs did not change time in the inflow zone, however, when looking across test solutions, RFs and NFs did not spend significantly more time in the inflow zone during the first 5 min stimulus period when exposed to any of the test solutions (H=6.87, P=0.23, and H=7.58, P=0.11 respectively) (Fig 10-13).  When a preparation containing 0.1 nM 11-O-ETIO-17-s, 11-O-ETIO-3-s, 11-

O-ETIO-17-g, and 11-O-ETIO-3-g (but not the free 11-O-ETIO) was introduced, RFs showed a delayed attraction to the inflow zone.

When comparing the change in the percent of time spent in the inflow zone between the last five minutes of the acclimation period and the first five minutes of the stimulus period, across test solutions, it was found that neither RFs nor NFs changed significantly (RF: H=7.49, DF=5, P=0.19, NF: H=4.50, DF =5, P=0.48, Fig 8). The differences in the change in percent of time spent in the inflow zone between RFs and NFs within each test solution were also not significant (Table 3.2) (Fig. 3.7).

**Change in Distance Travelled**

Change in distance travelled was not correlated to GSI ($r^2$= 0.01, P=0.48) (Fig. 3.7). When exposed to the vehicle blank (NF, n=5) 4 fish moved very little, and one decreased the distance travelled (Fig. 3.14). Most RFs and NFs increased distance travelled in unfractionated CW extract and in the fraction mixture lacking 11-O-ETIO-3-s (Fig. 3.14). Some fish increased movement, some did not change distance travelled and others moved less (Fig. 3.14). Reproductive females significantly decreased the distance travelled (boxes moved) during the first 5 minutes of the stimulus period when exposed to fractions 16-24 and 27-31 (containing 0.01 nM 11-O-ETIO, 11-O-ETIO-17-s, and 11-O-ETIO-3-s) (Minus 17-g and 3-g) when compared to the change in distance travelled when exposed to CW extract, but not to any other test solutions (H=12.82, DF=4, P=0.01, Fig. 3.15). NFs did not significantly differ in the distance travelled between the acclimation and stimulus periods when exposed to any of the test solutions (H=3.04, DF=4, P=0.55, Fig. 3.9). When comparing the change in distance travelled (between acclimation and

stimulus period) between RFs (n=13) and NFs (n=23) within each test, RFs decreased the

distance travelled significantly more than NFs (U=92.00, P=0.047) when exposed to a

steroid blend of 11-O-ETIO, 11-O-ETIO-17-s, and 11-O-ETIO-3-s (Fractions 16-24, and 27-

31 -Minus 17-g and 3-g) (Fig. 3.15).  There was no significant difference in change in

distance travelled between RFs and NFs in any of the other test solutions (Table 3.3)

(Fig. 3.15).  It should be noted that the power of the statistical tests used to compare

RFs and NFs when exposed to both minus 3-s and minus free as well as the change when

exposed to the vehicle blank were all below 0.8.

**Summary of Experiment 1 Findings**

Overall, the females did not show any strong responses to the test solutions

when comparing averages, but it may be informative to look at the changes in

behaviours of individual fish and look for trends.  When doing so, I found several trends

that support my predictions including: 1) when fraction pools that included 0.01 nM 11-

O-ETIO-3-s were applied, RFs tended to increase the time they spend in the inflow zone

(Fig. 3.8) and RFs did not change the percent of time spent in the inflow zone when the

test solution contained all fractions with the exception of 11-O-ETIO-3-s (Fig. 3.8), 2)

When fraction pools that included 0.01nM 11-O-ETIO-3-s were applied, RFs tended to

decrease movement (measured in distance) relative to NFs, whereas when 11-O-ETIO-3-

s was removed, RFs did not exhibit a change in distance travelled (Fig. 3.15).  In addition,

Previous observation that free 11-O-ETIO isolated from RM CW is not an attraction

pheromone (Tierney et al., 2013) to RFs is supported by our study, showing that RFs

displayed a delayed preference to a fraction mixture containing the 11-O-ETIO conjugates (but not free 11-O-ETIO).

## 3.2 EXPERIMENT 2: RESPONSES OF ROUND GOBIES IN TANKS WITH SHELTERS TO CONDITIONED WATER EXTRACT AND 1 $\mu$M SYNTHETIC STEROIDS

Female round gobies in tanks containing shelters (n=11) were exposed to CW extract containing 10 nM 11-O-ETIO or to 1 $\mu$M synthetic steroids (11-O-ETIO, 11-O-ETIO-3-s, 11-O-ETIO-17-s, and 11-O-ETIO-3-g). Each fish was exposed to four test solutions. The sequence for these tests was randomized for each fish. (1) CW extract, (2) a blend of 1 $\mu$M 11-O-ETIO, 11-O-ETIO-3-s, 11-O-ETIO-17-s, and 11-O-ETIO-3-g, (3) the blend in (2) without 11-O-ETIO-3-s, and (4) 1 $\mu$M 11-O-ETIO-3-s.

**Change in Percent Time Spent in Shelter**

RFs (n=5) and NFs (n=6) did not exhibit a significant change in the percent of time spent in the shelter when exposed to any of the test solutions (RF: F=0.14, P=0.93, NF: F=2.54, P=0.10) (Fig. 3.16 & 17). Nor was there a significant difference between RFs and NFs when exposed to each of the test solutions (Table 3.4) (Fig. 3.16 & 17). Fish generally remained in the tube for the entire duration of the experiments.

**Time Spent in the Inflow Zone**

Due to the small number of fish that actually entered the inflow zone, the data for each individual is expressed rather than as means (Fig. 3.18 & 19). When comparing the percent time spent in the inflow zone between the acclimation and stimulus period, no significant difference was found for RFs (n=5) or NFs (n=6) (Table 3.4) (Fig. 3.18 &19) (Table 3.5).

The amount of time spent in the inflow zone during the stimulus period did not differ significantly across each of the test solutions for either RFs ($\chi^2$=3.80, P= 0.284) or NFs ($\chi^2$=3.00, P=0.392) (Fig. 3.18 & 19). A similar result was found when comparing the time spent in the inflow zone across reproductive status during the stimulus period for each of the test solutions (Fig. 3.18 & 19) (Table 3.6).

Due to low response rates (1 RF and 1 NF entered the inflow zone) analysis of change in time spent in the inflow zone was not conducted

**Hopping Behaviour**

The number of hops over the acclimation and stimulus periods was measured as a metric of fish activity. When comparing the number of hops performed across time, it was found that NFs (n=6) hopped more during the stimulus period than the acclimation period when exposed to a blend of synthetic 1µM 11-O-ETIO, 11-O-ETIO-17-s, and 11-O-ETIO 3-g (Minus 3-s) (t=-4.00, P=0.01) (Fig 20C). There was a similar trend in response of NFs when exposed to 1 µM 11-O-ETIO-3-s (Z=2.00, P=0.06) (Fig. 3.20 D). There were no other significant differences when comparing hopping behaviour between acclimation and stimulus periods (Fig. 20 A-D). When exposed to a blend of synthetic 1 µM 11-O-ETIO, 11-O-ETIO-17-s, 11O-ETIO-3s and 11-O-ETIO 3-g (All steroids) RFs displayed a higher number of hops during the stimulus period than NFs (Table 3.7) (Fig. 3.20B). Reproductive females also performed a significantly higher hopping frequency during the stimulus period when exposed to a blend of 1 µM 11-O-ETIO, 11-O-ETIO-17-s, 11O-ETIO-3s and 11-O-ETIO 3-g (all steroids) compared to the hopping frequency during CW extract exposure (F=5.184, P=0.02), NFs did not exhibit significantly different hopping

frequencies during the stimulus period when exposed to any of the test solutions (F=0.34, P=0.80) (Fig. 3.21& 22).

Across tests of synthetic steroids, females (n=11) did not significantly differ in the change in hopping behaviour (F=1.50, P=0.24, Fig. 3.21 & 22). Data for reproductive and non-reproductive was pooled for this analysis as they demonstrated similar activity patterns (Mann-Whitney rank sum test comparing RF (n=43) and NF (n=48) change in hops: U=191.50, P=0.38) (Fig 13).

The response magnitude for hopping was greater than 2.5 for 2 NF (N=6) upon the delivery of the CW extract, of 1 μM 11-O-ETIO-3S, and of a mixture containing 1 μM 11-O-ETIO-17s, 11-O-ETIO-3-g and 11-O-ETIO (Fig. 3.21). For 1 RF (N=5), the response magnitude exceeded 2.5 in 11-O-ETIO-3-s (Fig. 3.21). The response magnitude was greater than 1 in all 6 NF tested, to a mixture of steroids that did not include 11-O-ETIO-3-s (but included 11-O-ETIO-17-s, 11-O-ETIO-3-g and 11-O-ETIO), in 4 NF tested with 11-O-ETIO-3-s, and 2 NF tested with all steroids. Two RFs showed a response magnitude greater than 1 in 11-O-ETIO-3-s; 3 in 11-O-ETIO-17-s, 11-O-ETIO-3-g and 11-O-ETIO, 2 in all steroids and 1 in CW extract. These findings indicate that hopping is greater in NFs than RFs, and that an elevated response magnitude was most frequent in the 11-O-ETIO-3-s treatment.

**Change in Gill Ventilation Rate**

Gill ventilation rate was measured to determine if females could detect the presence of the test solution (Murphy et al., 2001, Tierney et al., 2013). Across test solutions (synthetic steroids delivered at 1μM), Neither RFs (n=5) nor NFs (n=6)

significantly changed gill ventilation rates (RF: F=1.78, P=0.21, NF: (F=0.26, P=0.86) (Fig

23).  When comparing the change in gill ventilation across reproductive status, when

exposed to synthetic 11-O-ETIO-3-s RFs significantly decreased gill ventilation rate in

comparison to NFs.  Across other test solutions, no significant differences were found

(Table 3.8) (Fig. 3.23).

I also compared the gill ventilation rate per minute during the last 5 minutes of

the acclimation period to the first five minutes of the stimulus period using a paired t-

test.  I found no statistical differences between these time periods during application of

any of the test solutions.  Although there was a slight decrease in RF gill ventilation rate

(n=5) when exposed to synthetic 11-O-ETIO-3-s (1μM) (t=2.21, P=0.09) (Fig. 3.23).  The

power of all statistical tests used to examine changes in gill ventilation activity were

below 0.8.

**Table 3.2** Statistical data from Experiment 1, investigating the change in percent time in the inflow zone in response to various isolates of reproductive male round goby conditioned water

| Test Solution | Sample size RF | Sample size NF | U value | P value |
|---|---|---|---|---|
| CW with 10 nM 11-oxo-ETIO | 22 | 29 | 287.99 | 0.53 |
| Fractions 19-31 (0.01 nM 3S, 3g, 17g, free (Minus 17-s) | 10 | 19 | 70.50 | 0.25 |
| Fractions 16-21; 24-31(Minus 3-s) | 10 | 10 | 44.50 | 0.67 |
| Fractions 16-24; 27-41 (Minus 17-g and 3-g) | 11 | 18 | 81.00 | 0.41 |
| Fractions 16-26 (Minus Free) | 11 | 10 | 54.50 | 0.99 |

**Table 3.3** Statistical data from Experiment 1, investigating the change in distance travelled in response to various isolates of reproductive male round goby conditioned water

| Test Solution | N RF | N NF | U value | P value |
|---|---|---|---|---|
| CW | 13 | 23 | 108.00 | 0.12 |
| Minus 17-s | 12 | 23 | 97.50 | 0.38 |
| Minus 3-s | 11 | 13 | 28.50 | 0.79 |
| Minus 17-g and 3-g | 13 | 23 | 92.00 | 0.047 |
| Minus Free | 11 | 11 | 34.00 | 0.41 |

**Table 3.4** Statistical data from Experiment 2, investigating the time spent in the PVC shelter change in response to various synthetic analogs of reproductive male round goby conditioned water

| Test Solution | U value | P Value |
| --- | --- | --- |
| CW Extract | 22.00 | 0.18 |
| All Steroids | 12.00 | 0.66 |
| Minus 3-s | 7.00 | 0.18 |
| 3-s Alone | 14.00 | 0.93 |

**Table 3.5** Statistical data from Experiment 2, investigating the time spent in the inflow zone across the acclimation and stimulus periods in response to various synthetic analogs of reproductive male round goby conditioned water

| Test Solution | RF Test Statistic | RF P value | NF Test Statistic | NF P value |
| --- | --- | --- | --- | --- |
| CW Extract | Z=1.00 | 0.99 | Z=1.00 | 0.99 |
| All Steroids | t=1.65 | 0.20 | $Z=4.52X10^{-272}$ | 0.99 |
| Minus 3-s | Z=-1.00 | 0.99 | Z=-1.00 | 0.99 |
| 3-s Alone | $Z=-2.48X10^{-72}$ | 0.99 | Z=-1.00 | 0.99 |

**Table 3.6** Statistical data from Experiment 2, investigating the time spent in the inflow zone across the acclimation and stimulus periods between reproductive and non-reproductive females in response to various synthetic analogs of reproductive male round goby conditioned water

| Test Solution | U value | P value |
|---|---|---|
| **All Steroids** | 9.00 | 0.99 |
| **Minus 3-s** | 15.00 | 0.99 |
| **3-s Alone** | 15.00 | 0.99 |
| **CW Extract** | 14.00 | 0.93 |

**Table 3.7** Statistical data from Experiment 2, investigating the change in hopping frequency during the acclimation and stimulus periods in response to various synthetic analogs of reproductive male round goby conditioned water

| Test Solution | RF (n=5) | | NF (n=6) | | RF vs. NF | |
|---|---|---|---|---|---|---|
| | t value | P value | t value | P value | U value | P value |
| CW extract | 1.15 | 0.31 | -0.79 | 0.47 | 9.50 | 0.33 |
| All Steroids | 0.13 | 0.90 | $-1.22 \times 10^{-16}$ | 0.99 | 2.00 | 0.04 |
| Minus 3-s | -0.67 | 0.54 | -4.00 | 0.01 | 8.50 | 0.25 |
| 3-s Alone | -1.26 | 0.28 | 2.00* | 0.06 | 13.00 | 0.79 |

*did not pass normality, Wilcoxon signed rank test was used

**Table 3.8** Statistical data from Experiment 2, investigating the change in gill ventilation rate in response to various synthetic analogs of reproductive male round goby conditioned water

| Test Solution | t value | P value |
|---|---|---|
| All Steroids | 0.45 | 0.66 |
| Minus 3-s | -0.25 | 0.81 |
| 3-s Alone | -2.714 | 0.024 |
| CW Extract | -1.11 | 0.30 |

**Table 3.9** Statistical data from Experiment 2, investigating the change in gill ventilation rate between reproductive and non-reproductive females in response to various synthetic analogs of reproductive male round goby conditioned water

| Test Solution | RF (n=5) | | NF (n=6) | |
|---|---|---|---|---|
| | t value | P value | t value | P value |
| All Steroids | 0.94 | 0.40 | -0.56 | 0.60 |
| Minus 3-s | -1.05 | 0.37 | -0.29 | 0.79 |
| 3-s Alone | 0.31 | 0.77 | 0.14 | 0.89 |
| CW Extract | 2.01 | 0.09 | -1.58 | 0.18 |
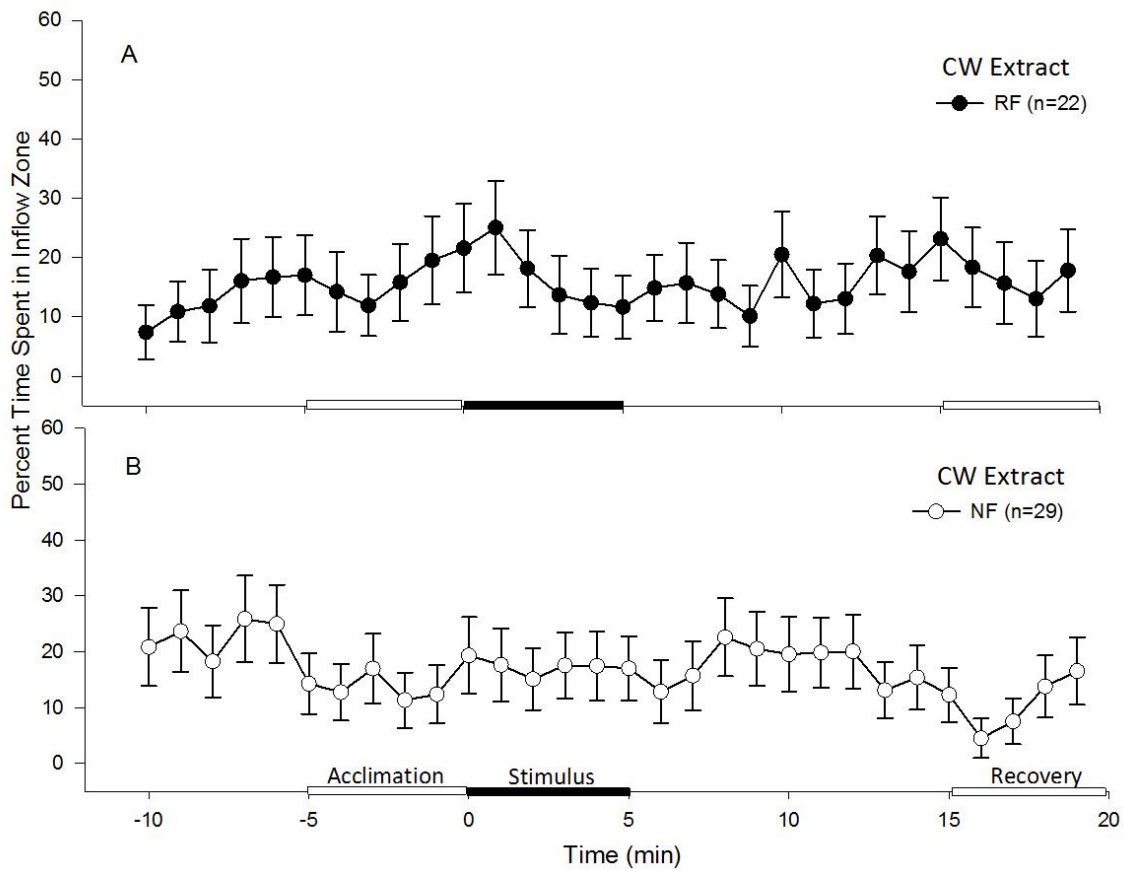
**Figure 3.6** Experiment 1, average percent time (± SEM) spent in the inflow zone per minute after delivery of conditioned water (CW) extract (10nM) of both (A) reproductive females (RFs) (closed circles) and (B) non-reproductive females (NFs) (open circles). The x-axis is time in minutes with negative values referring to the pre-odour delivery period. The bars along the x-axis represent each of the 5 minute time periods used for statistical analysis. No significant difference was found across reproductive status or time periods.
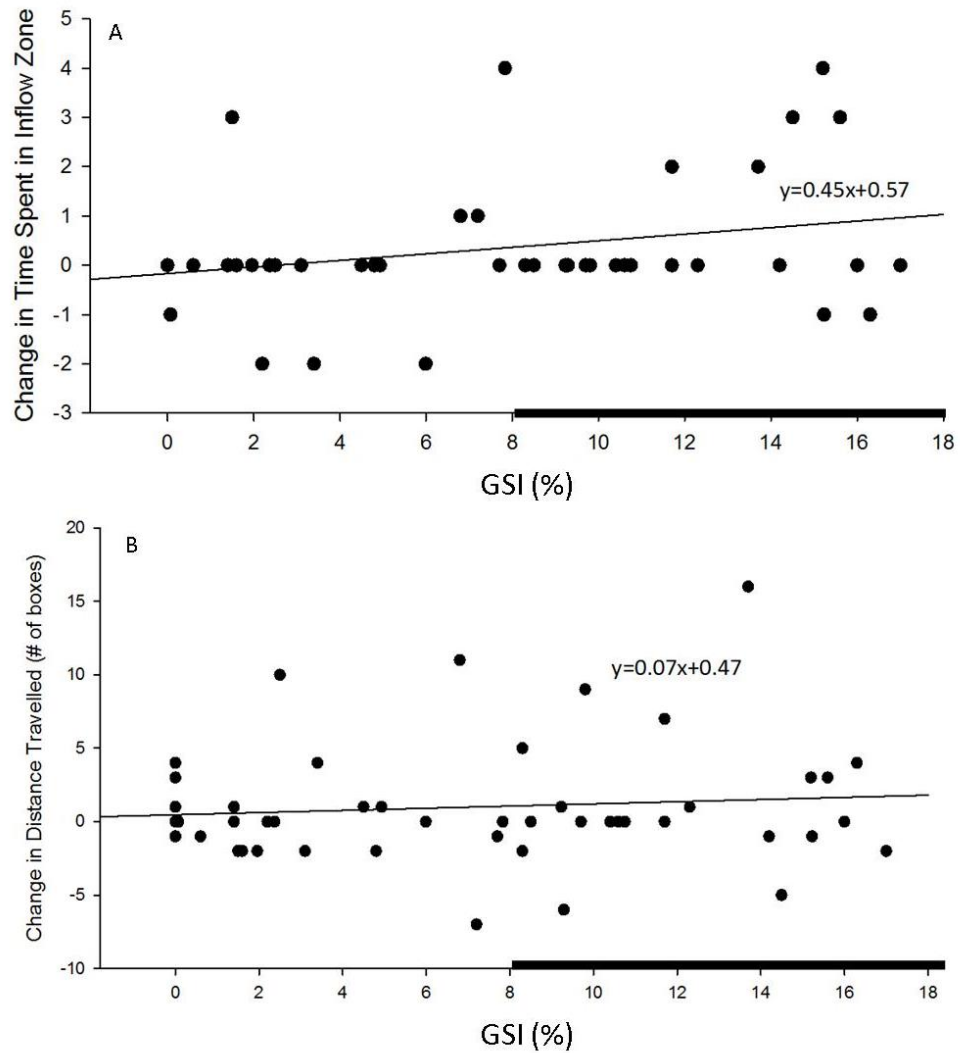
**Figure 3.7** Change in both time spent in inflow quarter (A) and distance travelled (B) calculated as: last 5 minutes during acclimation period subtracted from first 5 minutes of stimulus period, in relation to GSI when fish were exposed to CW extract ($10^{-8}$M) in experiment 1. The black bar along the x axis denotes reproductive females. Females with a GSI greater than 8.3 were considered reproductive (black bar along x-axis) (Gammon et al., 2005). There was no correlation found for either metric against GSI.
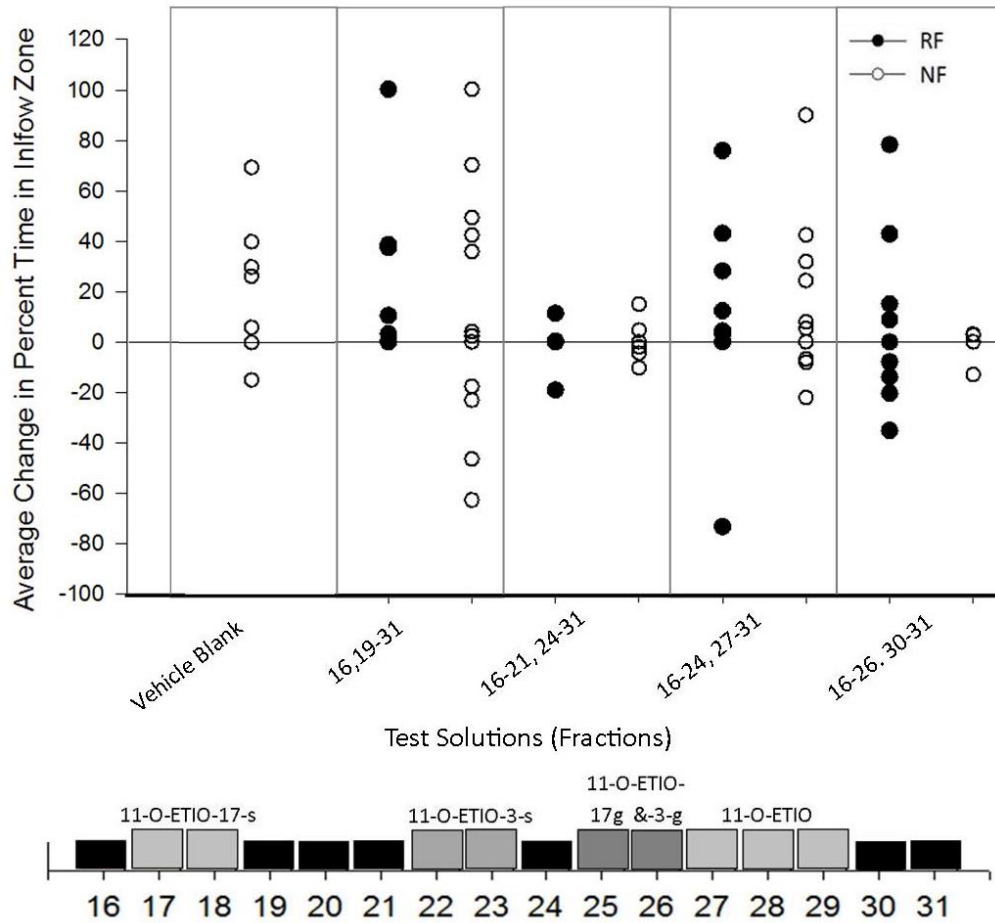
**Figure 3.8** Average change in the percent time spent in the inflow zone (± SEM) over five minutes for reproductive females (RFs, closed circles) and non-reproductive females (NFs, open circles) after test solution delivery of (i) vehicle bank, (ii) all fractions except for 17-s, (iii) all fractions except for 3-s, (iv) all fractions except for 17-g and 3-g, (v) all fractions except for free. There were no significant differences across treatments or reproductive status.
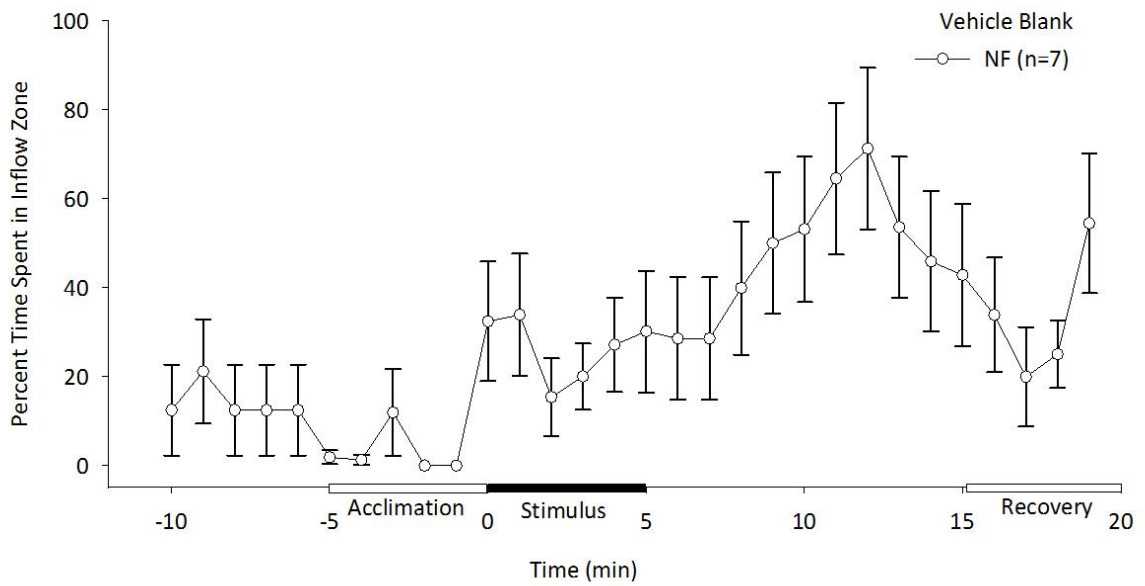
**Figure 3.9** Average percent time (± SEM) spent in the inflow zone per minute after delivery of vehicle blank. Fish spent significantly more time in the inflow zone during the stimulus and recovery periods when compared to the acclimation period (P=0.002)
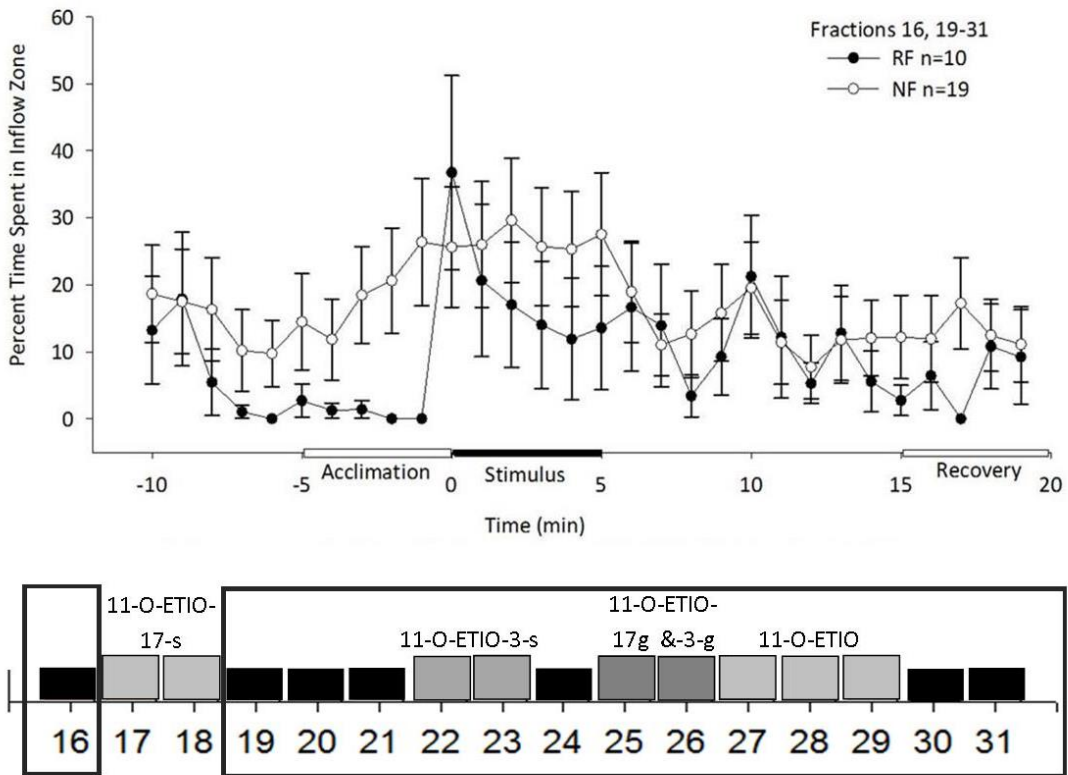
**Figure 3.10** Average percent time (± SEM) spent in the inflow zone per minute by RFs (closed circles) and NFs (open circles) after delivery of all Experiment 1 fractions except for those containing 11-O-ETIO-17-s. There was no significant difference found when comparing across time or reproductive status. The black boxes denote the fractions that were delivered.
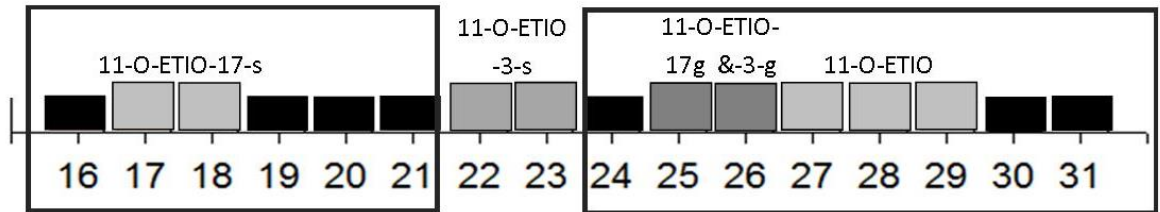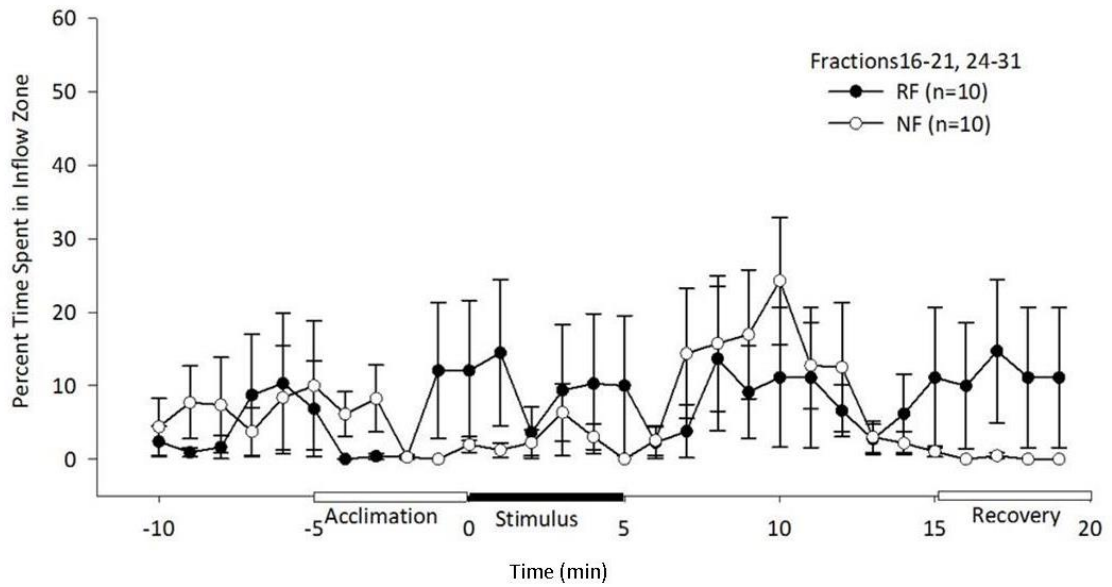
**Figure 3.11** Average percent time (± SEM) spent in the inflow zone per minute by RFs (closed circles) and NFs (open circles) after delivery of all fractions except for 3s. There was no significant difference found when comparing across time or reproductive status. The black boxes denote the fractions that were delivered.
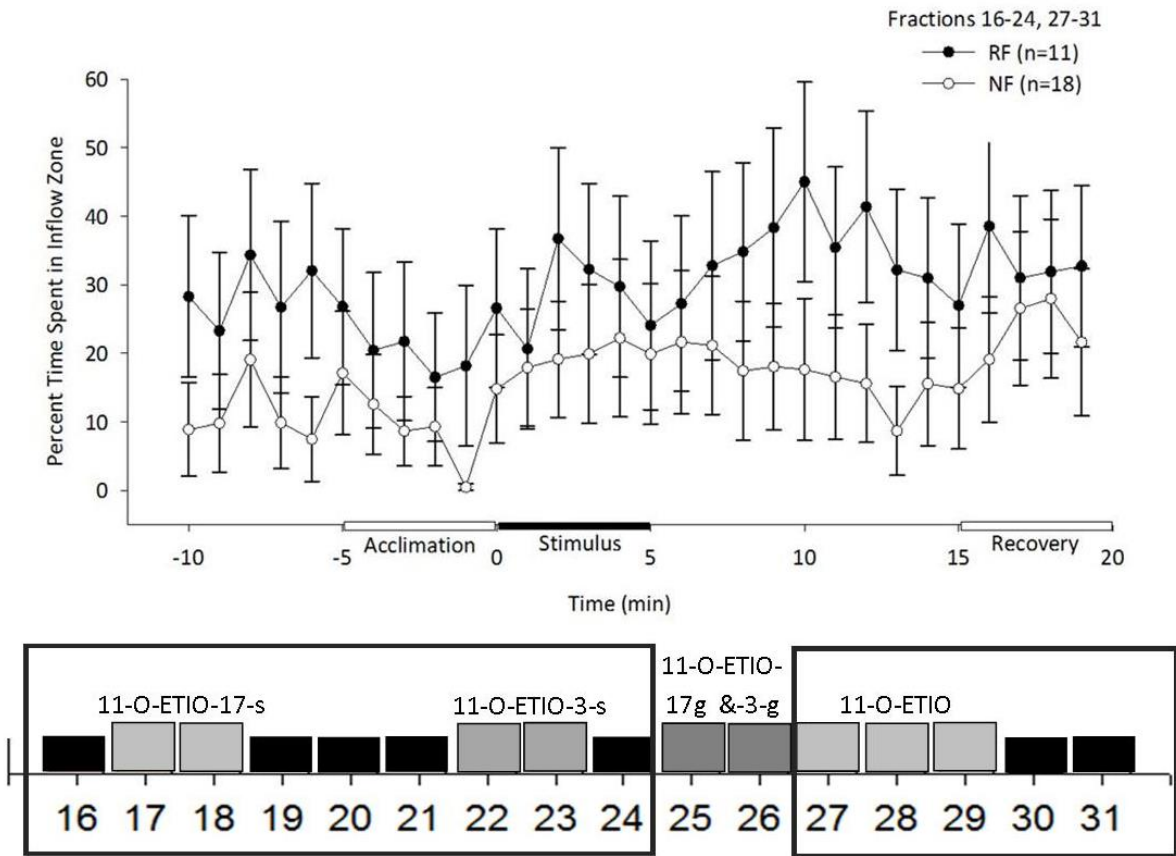
**Figure 3.12** Average percent time (± SEM) spent in the inflow zone per minute by RFs (closed circles) and NFs (open circles) after delivery of all fractions except for 17g and 3g, There was no significant difference found when comparing across time or reproductive status. The black boxes denote the fractions that were delivered.
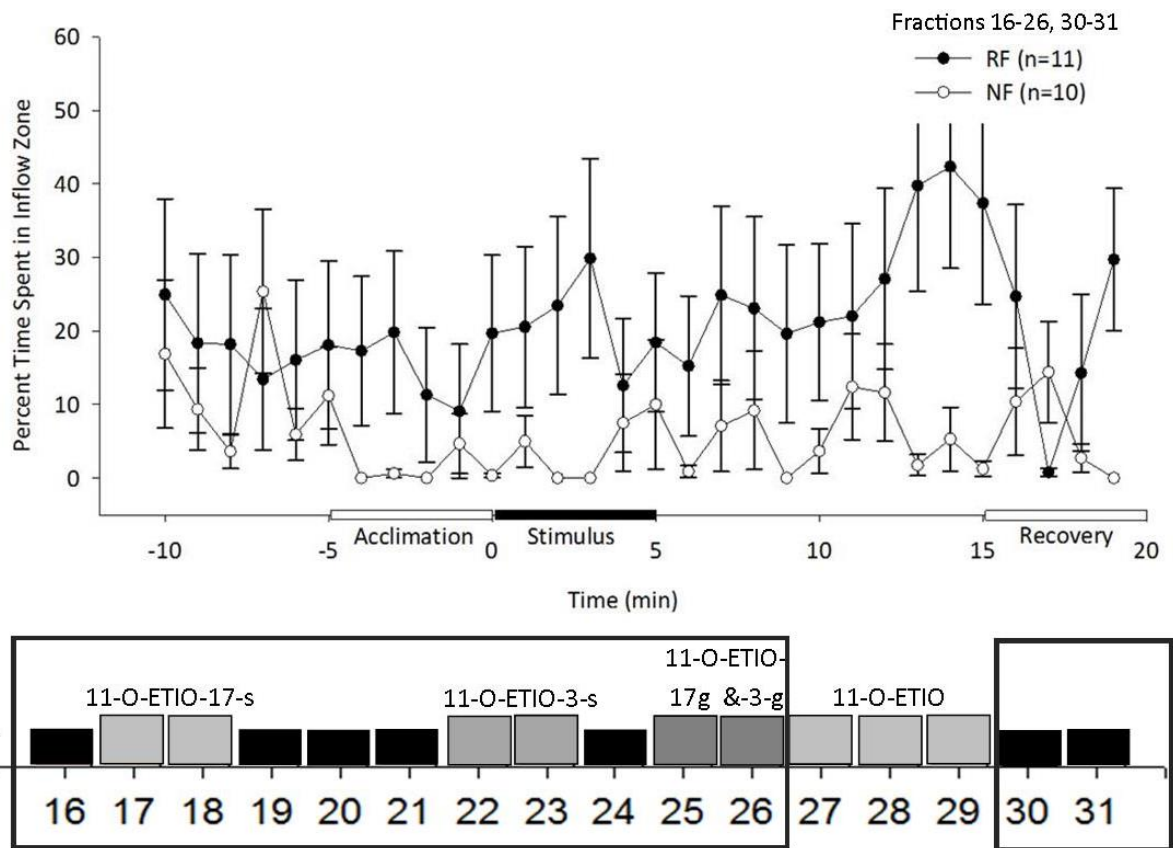
**Figure 3.13** Average percent time (± SEM) spent in the inflow zone per minute by RFs (closed circles) and NFs (open circles) after delivery of all fractions except for free. RFs spent more time in the inflow zone during the recovery period than the acclimation and stimulus periods (P=0.02). The black boxes denote the fractions that were delivered.
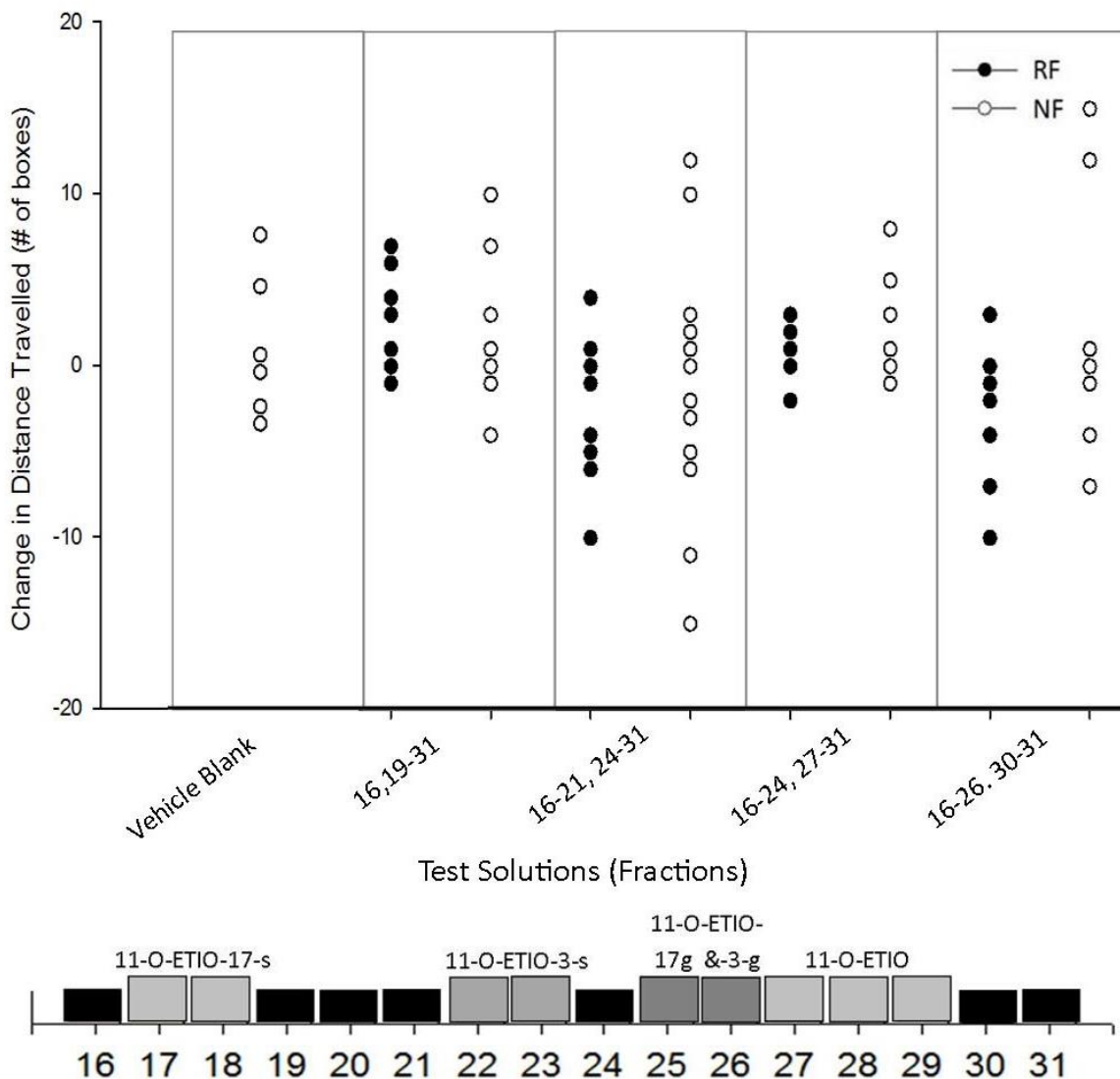
**Figure 3.14** Change in distance travelled (# of boxes ± SEM) of RFs (closed circles) and NFs (open circles) over the five minutes after test solution delivery compared to the five minutes before test solution delivery of (i) vehicle blank, (ii) all Experiment 1 fractions except for 17-s, (iii) all fractions except for 3-s, (iv) all fractions except for 17-g and 3-g, and (v) all fractions except for free.
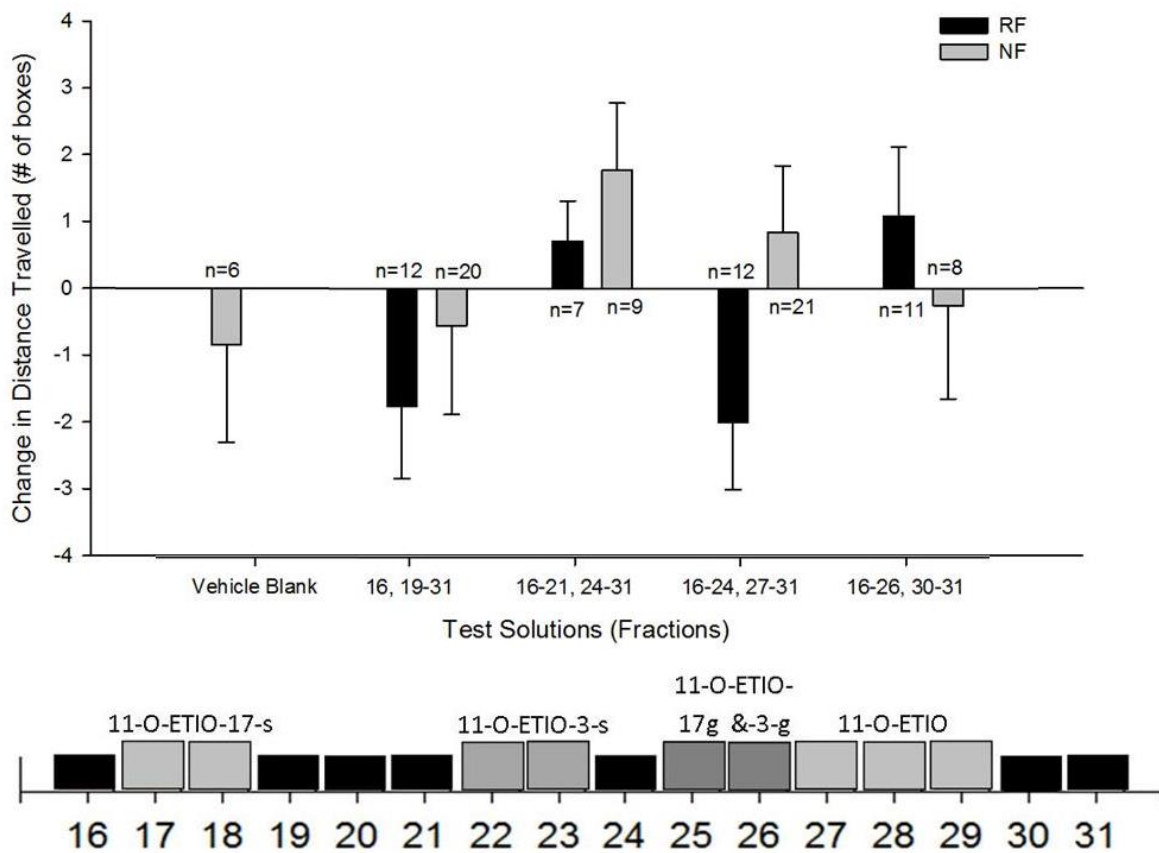
**Figure 3.15** Average change in distance travelled (# of boxes ± SEM) by RFs (black bars) and NFs (grey bars) over the five minutes after test solution delivery compared to the five minutes before test solution delivery of (i) vehicle blank, (ii) all Experiment 1 fractions except for 17-s, (iii) all fractions except for 3-s, (iv) all fractions except for 17-g and 3-g, and (v) all fractions except for free. RFs significantly increase their distance travelled more than NFs when exposed to all fractions except for 17-g and 3-g.
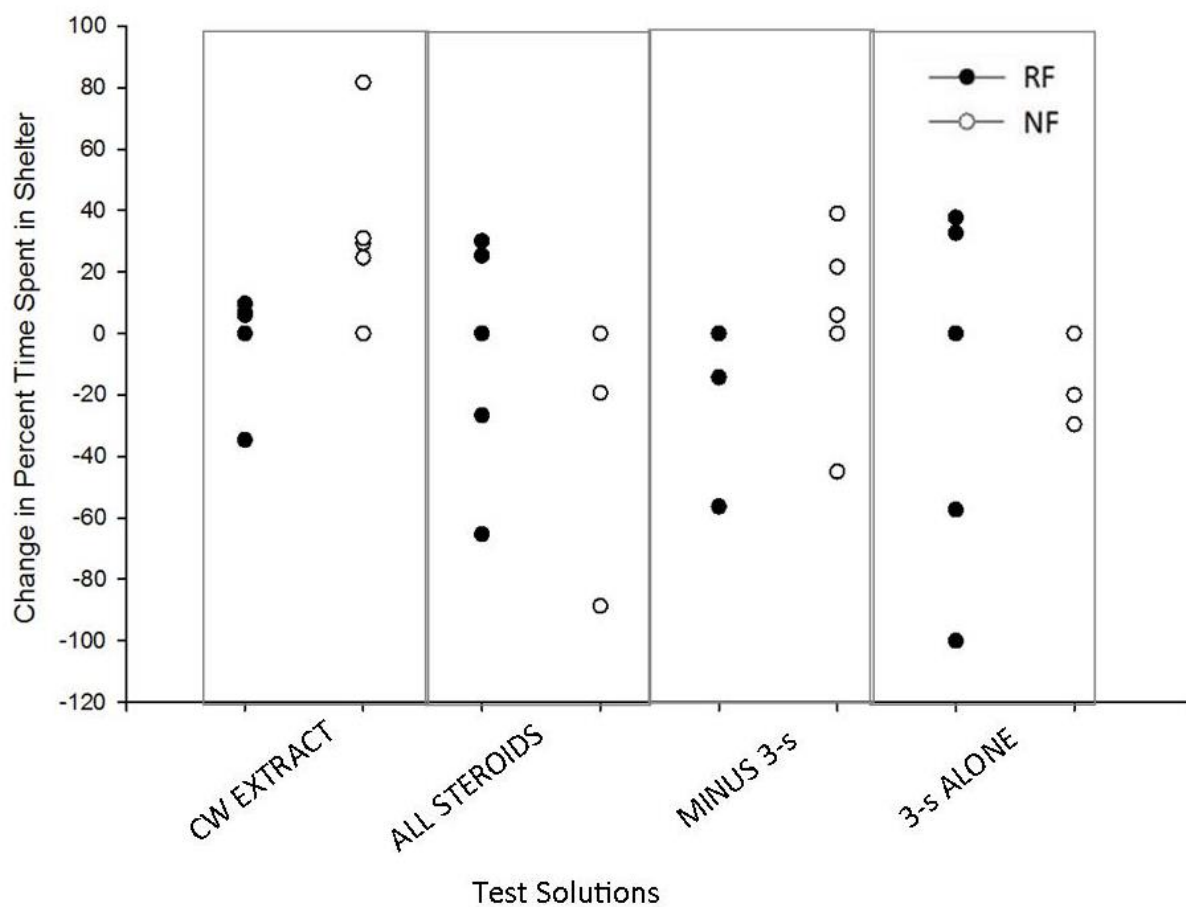
**Figure 3.16** Change in the percent time RFs (closed circles) and NFs (open circles), spent in the PVC shelter over five minutes after test solution delivery of (i) CW extract (10nM) and synthetic steroids at 1µM (ii) all steroids (11-O-ETIO, 11-O-ETIO-17-s, 11-O-ETIO-3-s, 11-O-ETIO-3-g) (iii) all steroids except for 3s, and (iv) 3s alone.
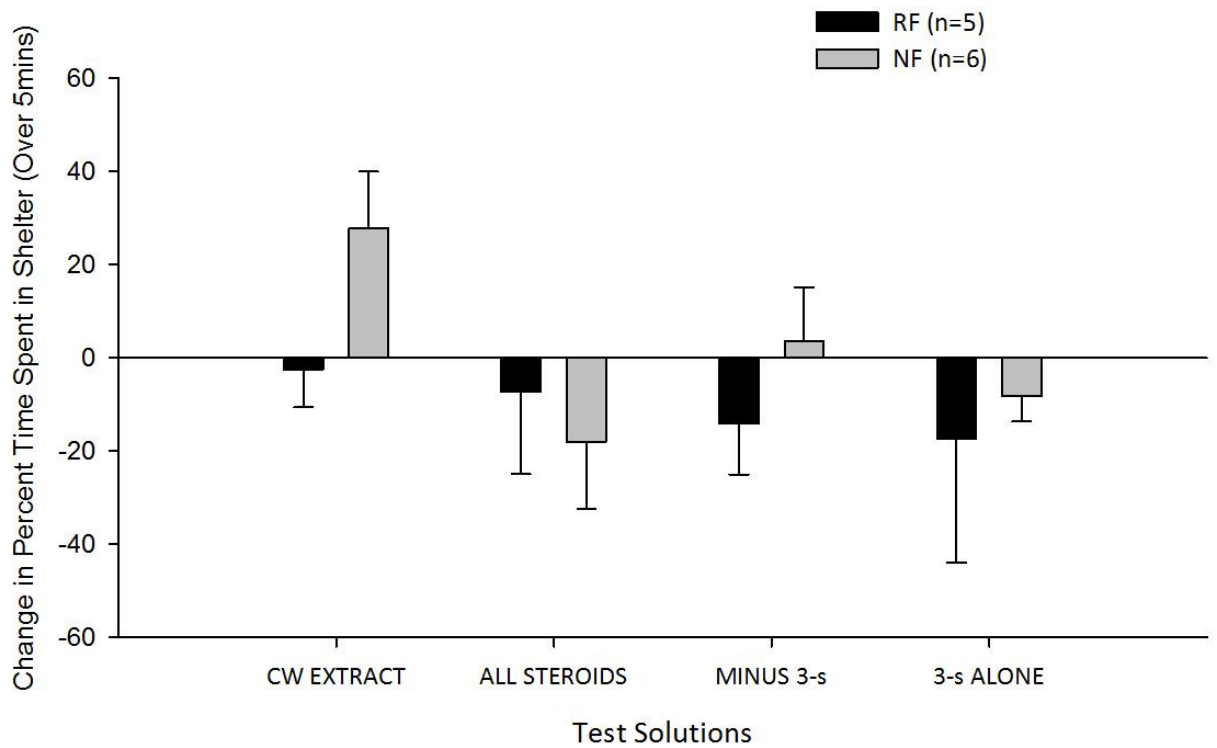
**Figure 3.17** Average change in the percent time RFs (black bars) and NFs (white bars), spent in the PVC shelter over five minutes after test solution delivery of (i) CW extract (10nM) and synthetic steroids at 1μM (ii) all steroids (11-O-ETIO, 11-O-ETIO-17-s, 11-O-ETIO-3-s, 11-O-ETIO-3-g), (iii) all steroids except for 3s, and (iv) 3s alone. There was no significant difference across any of the test solutions or reproductive status.
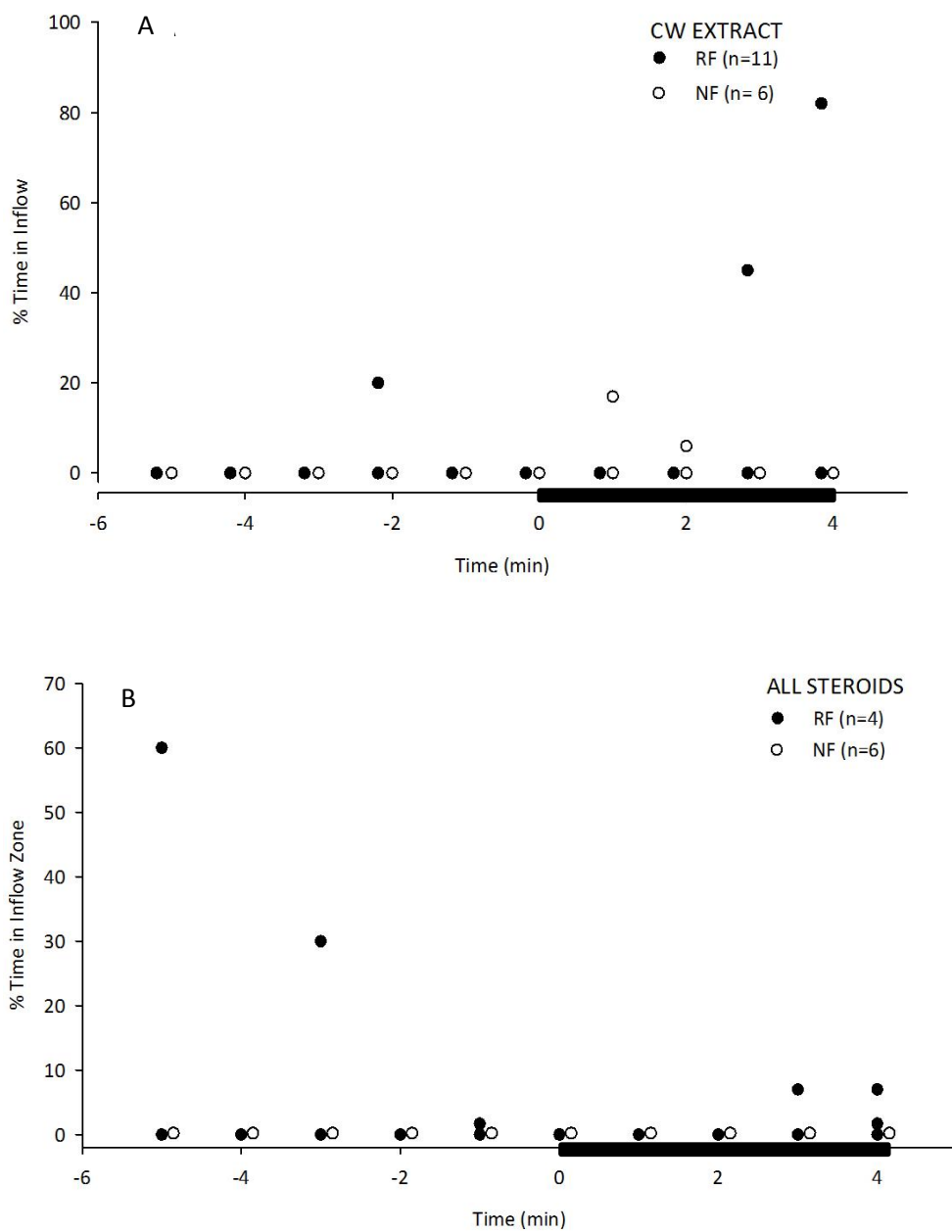
**Figure 3.18** Experiment 2 (with shelters), average percent time (± SEM) RFs (closed circles) and NFs (open circles) spent in the inflow zone per minute across treatments of synthetic steroids at 1µM after delivery of (A) CW extract ($10^{-8}$M), (B) all steroids (11-O-ETIO, 11-O-ETIO-17-s, 11-O-ETIO-3-s, 11-O-ETIO-3-g). The x-axis is time in minutes with negative values referring to the pre-odour delivery period. The black bar denotes when the test solution was in the tank.
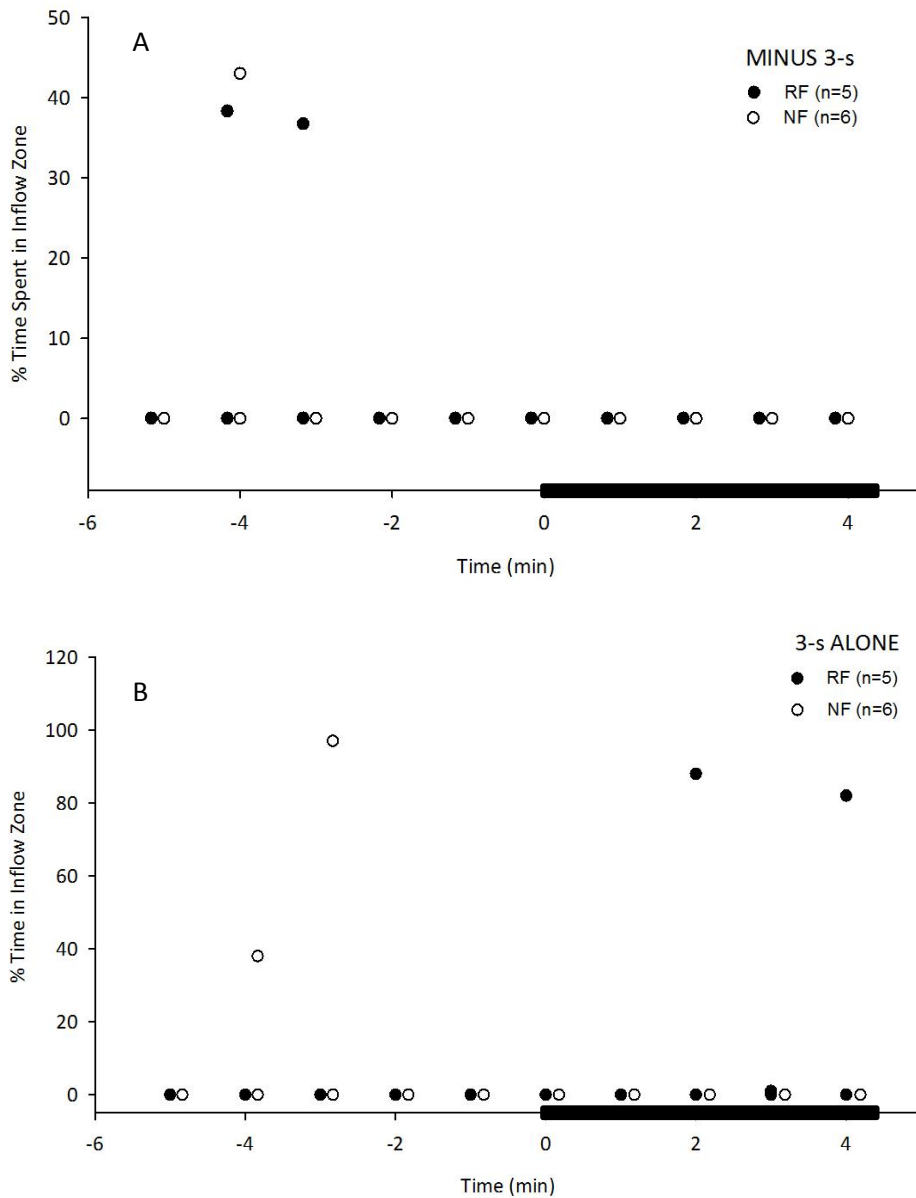
**Figure 3.19** Experiment 2 (shelters), average percent time (± SEM) RFs (closed circles) and NFs (open circles) spent in the inflow zone per minute across treatments of synthetic steroids at 1µM after delivery of (A) all steroids except for 3s, and (B) 3s alone.  The x-axis is time in minutes with negative values referring to the pre-odour delivery period.  The x-axis is time in minutes with negative values referring to the pre-odour delivery period.  The black bar denotes when the test solution was in the tank.
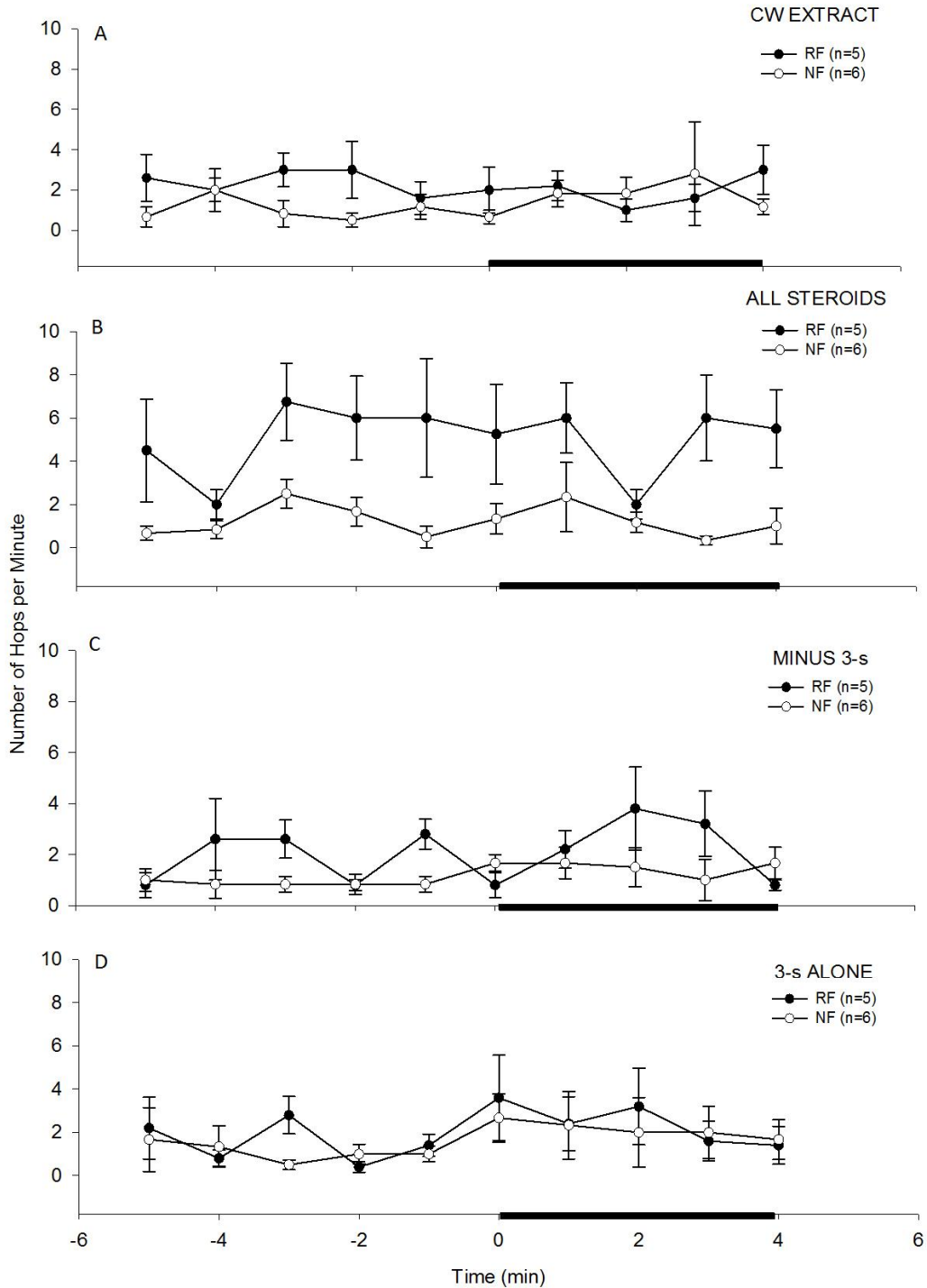
**Figure 3.20** Average number of hops (±SEM) across time RFs (closed circles) and NFs (open circles) performed per minute across treatments after delivery of (A) CW extract (10nM) and synthetic steroids at 1μM, (B) all steroids, (C) all steroids except for 3s, and (D) 3s alone. NFs significantly increased in number of hops in the inflow zone during exposure to steroid blend without 11-O-ETIO-3-s when compared to acclimation period.
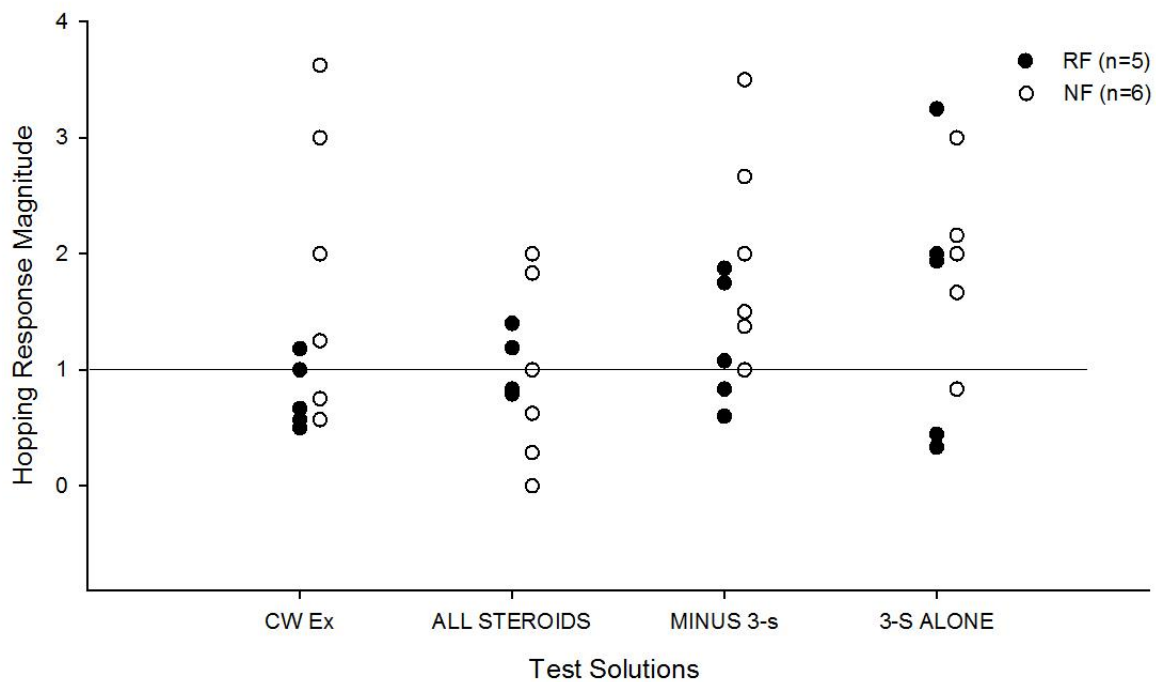
**Figure 3.21** Ratio of response for hopping activity (number of hops five minutes after solution delivery divided by the number of hops five minutes before delivery, exhibited by RFs (closed circles) and NFs (open circles), after delivery of (i) CW extract (10nM) and synthetic steroids at 1μM (ii) all steroids, (iii) all steroids except for 3s, (iv) and 3s alone.
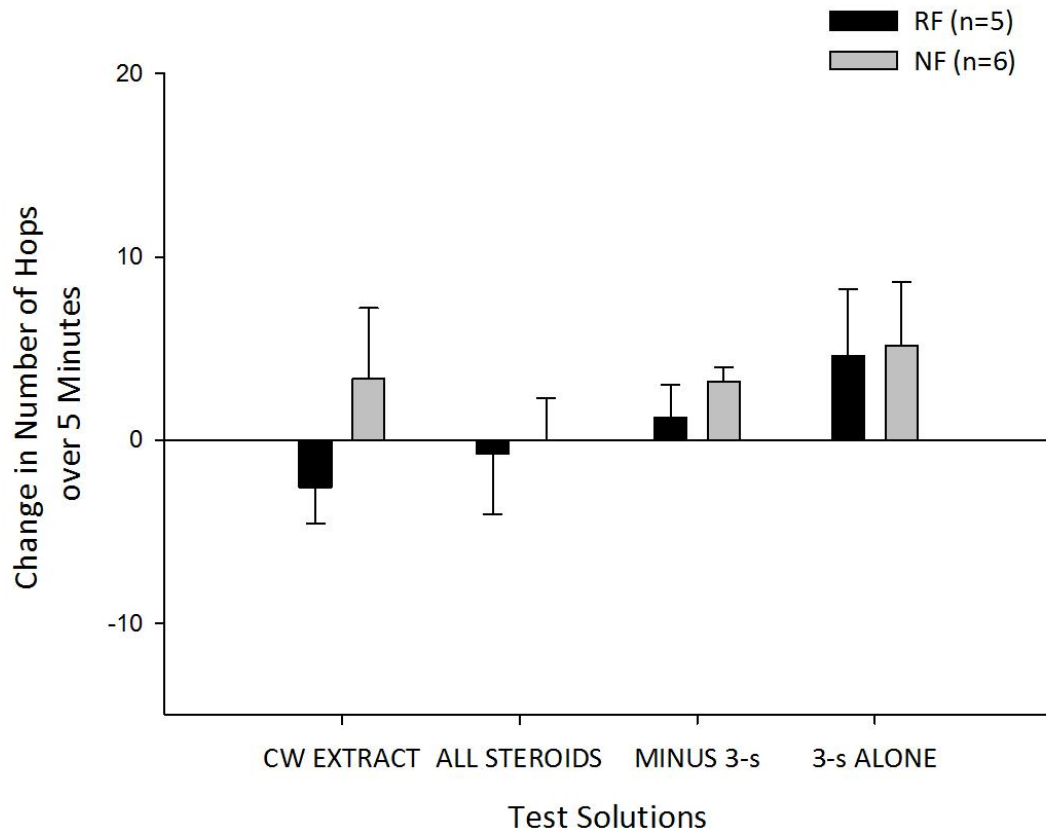
**Figure 3.22** Average change in hopping activity (number of hops five minutes after odour delivery minus number of hops five minutes before delivery, (± SEM) exhibited by RFs (black bars) and NFs (grey bars), after delivery of (i) CW extract ($10^{-8}$M) and synthetic steroids at $10^{-6}$M (ii) all steroids, (iii) all steroids except for 3s, (iv) and 3s alone. There were no significant differences across test solutions or reproductive status.
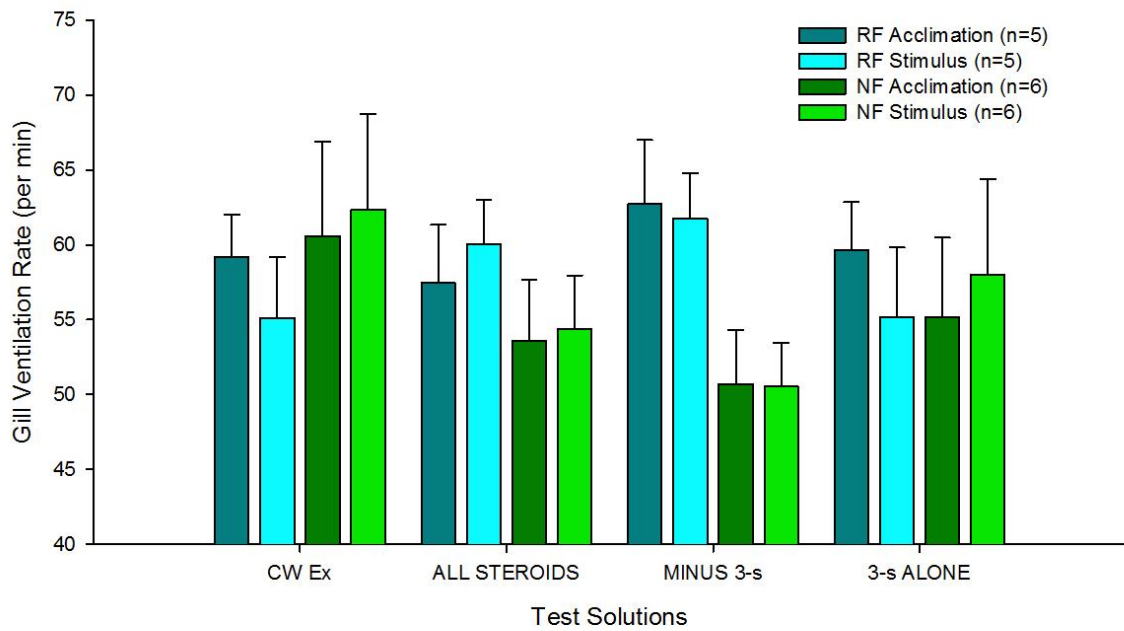
**Figure 3.23** Average gill ventilation rate (ventilations/minute) of RFs and NFs during the last five minutes before (acclimation) and first five minutes after (stimulus) test solution delivery (±SEM) after the delivery of (i) CW extract ($10^{-8}$M) and synthetic steroids at $10^{-6}$M, (ii) all steroids, (iii) all steroids except for 3s and (iv) 3s alone. There was no significant difference across any of the test solutions or reproductive status.

**4. DISCUSSION**

4.1 GENERAL DISCUSSION

Overall, female round gobies did not show significant movement responses to fractionated 0.1nM 11-O-ETIO isolates from RM CW or to 1μM synthetic analogs of these steroids. In the following sections I discuss some of the behavioural changes found in this study as well as describe possible reasons as to why most of the females did not respond to the test solutions.

In Experiment 1, I tested for changes in the movement of female round gobies, when pooled fractions containing 0.01 nM 11-O-ETIO derivatives and free 11-O-ETIO were applied to fish tanks. I predicted that RFs would stay near the inflow source and decrease distance travelled in tests containing 11-O-ETIO-3-s but overall my data did not support these predictions. One of the most likely reasons is the possibility that the steroids delivered were below the females olfactory threshold (Based on an EOG study) (Laframboise and Zielinski, 2011). Although it should be noted that the females used in that study were non-reproductive and it possible that olfactory receptor expression is higher in reproductive females as seen in African cichlids (*Astatotilapia burtoni*) (Marusa and Fernald, 2010). If this is the case, RFs may have a lower olfactory threshold for reproductive odours than NFs. It is also possible that the threshold for behavioural responses is below the threshold required to elicit an olfactory response in an EOG. This is the case in sea lamprey as females show an EOG response threshold to a male mating pheromone (3KPZS) delivered at $10^{-12}$M (Siefkes and Li, 2004 ), but have been shown to respond behaviourally to 3KPZS at $10^{-13}$M and in some cases $10^{-14}$M (Johnson et al.,

2009). In addition, because of the large number of non-responsive fish, it is possible a test solution may have been attractive but the response was not detected through the use of means. It should be noted that I did not directly test the olfactory capabilities of the fish used in my study.

In Experiment 2, gobies were provided shelters and responses to $1\mu M$ synthetic steroids were tested. In all tests, gobies tended to stay in the shelter, and NFs increased their time in the shelter when CW extract was delivered, however RFs seem to leave the shelter more when the steroids were delivered, especially in the presence of $1\mu M$ 11-O-ETIO-3-s alone (Fig 16). Hopping frequency was also quantified as an activity metric. Most RFs and NFs increased hops in response to 1 $\mu M$ 11-O-ETIO-3-s and in a mixture of 1 $\mu M$ 11-O-ETIO-17-s, 11-O-ETIO-3-g and free 1 $\mu M$ 11-O-ETIO (Fig. 3.21 and Fig. 3.22). Although the largest hop increase was seen in 1 $\mu M$ 11-O-ETIO-3-s, this value was not statistically different from baseline (Fig. 3.21). I found that RFs hopped more in response to test solutions containing the synthetic steroids ($1\mu M$ 11-O-ETIO, 11-O-ETIO-17-s, 11-O-ETIO-3-s, and 11-O-ETIO-3-g) than NFs. This observation should be interpreted cautiously as there was not a significant increase in RF hopping frequency between the acclimation and stimulus period. Reproductive females exhibited a high hopping frequency throughout that trial even before the odour was added, suggesting that the hopping behaviour was not linked to the test solution. I also found that NFs increased hopping frequency in response to a blend of synthetic 1 $\mu M$ 11-O-ETIO, 11-O-ETIO-17-s, 11-O-ETIO-3-s, and 11-O-ETIO-3-g. Although this is the first study to quantify hopping, a previous study has reported increased movement in CW extracted

unseparated 11-O-ETIO conjugates (Tierney et al., 2013). From our study it seems possible that NFs within the confined testing apparatus can smell but are not necessarily attracted to this steroid blend. Reproductive females did not respond significantly to any of the synthetic test solutions. Again these results should be interpreted cautiously as the round gobies did not respond to the positive control, the CW extract.

This study has expanded upon previous research suggesting that female round gobies are attracted to water conditioned by RMs (Gammon et al., 2005), and that conjugated 11-O-ETIO specifically, is attractive to RFs (Tierney et al., 2013). The present study used the amount of time spent near the odour source, distance travelled, activity (hops) and gill ventilation as behavioural metrics to quantify attraction to particular test solutions which have all been commonly used in round goby behaviour (Murphy et al., 2001; Gammon et al., 2005; Belanger et al., 2006, Tierney et al., 2013). Our test solutions used expanded upon previous research, CW extracts have been shown to evoke attraction in female round gobies (Kasurak et al., 2012) and mixes of conjugated 11-O-ETIO are attractive to RFs, whereas unconjugated 11-O-ETIO is attractive to NFs (Tierney et al., 2013). In terms of CW extract, this was previously tested by Kasurak and colleagues (2012) in a larger flume setting. They found that fish were only responsive during the month of July; this differs from Kereliuk's work which tested females throughout the breeding season (June-September) (2009). It is possible that by removing all polar substances from the CW via methanol extraction, priming pheromones are removed, or non-polar compounds necessary for eliciting responses in females are removed, and only in the height of the breeding season will elicit a

response. It is also possible that seasonal variation in environmental factors such as temperature, day length, and light intensity may play a role in female responsiveness to male pheromones (as reviewed by McNeil, 1991).  In the present study, I did not find a trend suggesting that the change in time spent in the CW extract inflow zone increased in relation to GSI, although it does appear as though there is a slight increase in time spent in the inflow zone as GSI increases. Based on previous work, it may be important to use females with a high GSI value (>10) when conducting future test (Marenette & Corkum, 2008).  Responses to RM CW are status dependent (black goby, *Gobius niger*, Columbo et al., 1980; round goby, Tierney et al., 2003; Zeyl et al., 2014).  These studies suggest status-dependent changes in olfactory sensitivity exist so that males only attract females ready to spawn.

In the following sections I discuss the non-significant results of each method of behavioural analysis for both experiments and provide possible explanations and solutions:

4.2 EXPERIMENT 1 (0.1 NM FRACTIONATED 11-O-ETIO CONJUGATES)

The time periods analyzed were over the five minutes in which the test solution existed in a concentration gradient (as per Gammon et al., 2005; Kasurak et al., 2012; Tierney et al., 2013).  It is possible that females could be responding within the first moments of detection and then become habituated.  When animals navigate in odour plumes, they will exit and re-enter the plume in order to maintain sensitivity and tracking ability (Vickers, 2000).  I also found that when exposed to minus free and minus

17g and 3g, RFs showed a slight delayed response. This is likely due to normal fluctuations in base line activity and not a residual response as the test solution after 10 minutes post-delivery would be below the round gobies detection threshold (Laframboise and Zielinski, 2011).  It is important to note that the overall percent time spent in the inflow zone in Experiment 1 was approximately 25%, a value expected by chance as the inflow zone comprises 25% of the tank.  Overall, the large variation between individuals is likely an important factor causing the change in percent time data to be non-significant.  The power of many of the performed statistical tests was below the desired 0.80 (reviewed by Jennions & Møller, 2003).  This seems to be a typical problem not only in round goby research (Gammon et al., 2005, Tierney et al., 2011) but in animal behavior studies in general (Jennions & Møller, 2003; Sih et al., 2004). Future studies would need larger sample sizes to overcome this issue.  The relatively large proportion of gobies that did not move during the tests greatly affected the statistical significance.  A better process to remove non-responders could also be implemented, perhaps by first testing fish with a positive control such as L-alanine (food odour) which would allow researchers to avoid non-responsive fish.  These differences could be due to differences in fish 'personalities' where some fish are more bold than others and exhibit higher levels of exploration and recover from startling events faster (Sih et al., 2004).

4.3 EXPERIMENT 2 (SHELTER PROVIDED, 1μM SYNTHETIC STEROIDS TESTED)

Once the PVC shelter was added, fish spent much less than the expected 25% of the time in the inflow due to fish spending most of their time in the shelter.  The benefit of this is that if the fish does spend any time in the inflow zone it is more likely correlated to attraction as it is less likely that they would leave the tube unless attractive odour (i.e. for food odours, Chapter 2).  Unfortunately because of this, the variation was quite large (all or none response), thus to better look at the data, a true positive control should be used in advance of trials to remove non-responders.  Although not significant, when looking at time spent in the shelter, RFs didn't seem to leave the shelter when exposed to CW, but NFs did spend more time *in* the shelter when exposed to it.  Alternatively RFs did leave the shelter more in response to the synthetic test solutions (although not significant), suggesting that the micromolar steroids could elicit stronger responses than the 0.1 nanomolar values administered in the fractionated CW extract tests.  Gammon and colleagues (2005) also used a shelter, and observed that females spent approximately 20-25% of the time in the inflow half during the acclimation period, which is less than expected by chance (50% of the time).  This supports the idea that when a PVC shelter is available, fish spend more time in it, rather than in open areas.  Despite this, when RFs were exposed to RM CW they spent significantly more time in the inflow zone than in the acclimation period (81% of the 15 minute stimulus period) (Gammon et al., 2005).  This supports the idea that females leave the tube if they smell an attractive odour.

**Change in Distance Travelled**

Individual variation in change in distance travelled data was not as extreme (due to the fact that data was inherently normalized for each fish). In the case of distance travelled, the lack of significant changes could be a result of the small tank size used as a test for movement responses. In order to locate an odour source, fish will often navigate within the plume in order to best detect the areas of high concentration (Vickers, 2000). It is possible that in these smaller tanks, the fish are not able to perform this behaviour as they are essentially always in the high concentration zone. This was seen in previous work using these small tanks (Tierney et al., 2013). In that study, the authors report a decrease in distance travelled in the presence of round goby pheromones, which is atypical for these types of studies which typically see an increase in movement in response to pheromones (Johnson et al., 2009).

**Hopping Frequency**

It is possible that distance moved is not the most ideal metric for this study as the arena is small relative to the fish and gobies generally do not exhibit typical swimming behaviour, rather they tend to travel in short bursts or hops (pers. obs.). Thus, hopping may be a more appropriate metric for measuring changes in activity levels in response to a stimulus. In Experiment 2, hopping behaviour was used as a metric for activity. However, fish did not change hopping activity in response to any of the test solutions. There was a large variation in the baseline hopping ratio (during acclimation period, some fish hop constantly whereas others do not hop at all) I tested the hopping response ratio in order to control for individual variation and saw that RFs

increased hopping ratio in 1 μM 11-O-ETIO 3-s. I predicted that 11-O-ETIO-3-s conveys

odor potency based on previous EOG studies (Laframboise and Zielinski, 2012). Further

studies are needed as this was not statistically significant, likely due to low sample size.

Interestingly, NFs showed a large increase in hopping behaviour (2-3 fold increase from

acclimation) in CW, minus 11-O-ETIO-3-s and 11-O-ETIO-3-s alone but not to all steroids.

This is unexpected as NFs did not typically respond to CW or conjugated steroids but

instead preferred free (unconjugated 11-O-ETIO) (Corkum et al., 2008; Tierney et al.,

2013). Because hopping behaviour is not necessarily linked to attraction, it could

provide evidence in support of the idea that NF actually avoid RM odours (Gammon et

al., 2005).

**Gill Ventilation Rate**

Gill ventilation was expected to increase in response to CW and any solutions

that fish can smell (Belanger et al., 2006; Tierney et al., 2013), however I did not see any

significant changes in gill ventilation rates across treatments. Within treatments, RFs

showed a slight decrease in gill ventilation rate when exposed to 1μM synthetic 11-O-

ETIO-3-s, when comparing to rate before test solution delivery. For the present study,

gill ventilation rate was measured over a 10 second time period, every 30 seconds; this

value was then averaged over the 5 minute time period (acclimation vs. stimulus). It is

possible that the 10 second time bins were too short to determine an accurate gill

ventilation rate and that changes in gill ventilation rate may be short lived and thus, by

averaging over five minutes, small changes are missed. A previous study that focused

on gill ventilation rate changes in response to round goby pheromones used a similar method but calculated values for an entire 1 minute interval every 3 minutes (over a 15 minute time period) and then averaged these three values (Belanger et al., 2006). In that study, they also expressed changes as a percent increase from baseline (the acclimation period prior to the test solution delivery. From this, it is likely that the 10 minute time bins were inadequate for determining gill ventilation rate, and future studies should aim to use an entire 1 minute interval.

4.4 SUMMARY OF FINDINGS

**1) Concentration:** In Experiment 1, the steroid concentration in the HPLC fractions was below the round goby's olfactory detection threshold. The concentration that was tested was no higher than 0.1nM for each 11-O-ETIO conjugate. Females showed an olfactory threshold (by electro-olfactogram) for 1 nM ETIO and 1 nM 11-O-ETIO-3S (Laframboise and Zielinski, 2011).) . In Tierney and colleagues (2013), the fractions contained approximately 10 nM conjugated 11-O-ETIO.

**2) Positive Control:** While I did not see changes in movement when RM CW extract (containing 1 nM 11-O-ETIO) was delivered; this test solution was linked to movement responses in previous studies (Kasurak et al., 2012; Tierney et al., 2013).

**3) Synthetic Steroids:** It is possible that the mixtures of all steroids did not elicit a response because of the use of 11-O-ETIO-3-g as a racemic mixture, or because 11-O-ETIO-17-g was not included in the test solution (not commercially available). 11-O-ETIO-3-s did seem to elicit a higher hopping response magnitude in some RFs.

**4) The Ratio of Steroids:** The ratio of steroids tested could differ from that released by the male due to variation in isolation efficiencies of each of the steroids. The different structural groups on the conjugated steroids will affect the compound's polarity and thus its binding affinity in both the C18 column and the HPLC column. If the ratio varies drastically from that released by the male, it may affect the response rates. This would also affect the responses in the synthetic steroids experiment as the ratios used for that were based on those determined by HPLC.

**5) Circadian Rhythm**. Tierney and colleagues (2013) (after which the present study is modeled) ran experiments in the evenings in the dark. The experiments reported in the present study were performed in the afternoon under a low light setting in order to mimic light setting of shallow water. A study on swimming performance in the round goby suggests a slight bias towards nocturnal activity (Tierney at al., 2011). Unfortunately most studies reported in published literature do not mention the time of day the experiments take place (Murphy et al., 1999, Gammon et al., 2005; Corkum et al., 2008; Kasurak et al., 2012) so it is difficult to verify if time of day is important in the round goby and further studies are required to elucidate the full effects of time of day on behavioural responses to pheromones. In their review, Johnson and Li (2010) suggest that a variety of environmental cues including lighting, time of year, and hydrodynamics can play an important role in fish responses to olfactory cues.

**5. Conclusions & Recommendations for Future Studies:**

Overall, due to small sample sizes and low concentrations of steroids within HPLC

fractions, as well as the lack of response of females to CW extract (which I expected to elicit a

response), the data presented in Chapter III should be interpreted with caution.  Despite these

issues, there does appear to be some consistent evidence across Experiment 1 and 2 that

suggests that 11-O-ETIO-3-s is important in round goby sexual signaling.  It is unlikely that 11-O-

ETIO-17-s an attractive component, but the importance of the glucuronated 11-O-ETIO

requires further testing.  Based on our results it is possible that 11-O-ETIO-3-s could be used to

attract females to traps, but further testing is required.  Suggestions regarding the

experimental designs to consider for testing round goby movement responses are

presented in Chapter II of this thesis. Future studies on female movement responses to

reproductive steroids in the round goby should ensure concentrations delivered are

within the olfactory detection threshold.  In addition, due to the large variation in

behaviours, sample sizes greater than that used in this study would strengthen the

statistical power of the analysis. I also recommend the use of precursor test to eliminate

non-responsive fish as well as pretesting with a positive control for CW extract and a

negative control using a vehicle blank in order to provide baseline levels of activity of

each fish.

## CHAPTER IV- CONCLUSIONS & RECOMMENDATIONS

Overall this thesis provides a review of several options for testing round goby movement responses to odors (including putative pheromones), as well as supporting preliminary evidence for pheromonal function for 11-O-ETIO-3-s.  In Chapter II, I reviewed nine studies that studied round goby behaviours in response to olfactory cues in an effort to determine potential causes for inconsistencies in the results of these studies.  I focused the review on the 5 most important (and variable) factors across these experiments: apparatus type, flow rate, tank enhancements, behavioural metrics, and sample size.  I examined each of these factors within the nine studies and provide recommendations for future studies based on round goby life history as well as evidence from other species.  In addition I presented novel data to further test the importance of tank enhancements as this had not been tested previously.

I proposed that the apparatus type remain variable as it should be chosen to best answer the proposed question.  The flow rate typically used in these experiments is lower than what females likely encounter in the wild, and previous studies suggest that females prefer flow rates in the order of 100mL/min.  I suggest that future studies aim to have a flow rate within the range of those that females encounter in the field.  Tank enhancements (a PVC shelter, and gravel substrate) were shown to decrease the activity of females, but also made the detection of responses more clear.  I proposed that PVC shelters, but not gravel substrate, may enhance female responses and should be considered when designing studies such as those reviewed.  The types of behavioural

metrics used in the reviewed studies were fairly consistent, but the use of distance travelled yielded contradictory results. Time spent in the inflow zone was one of the most informative and appropriate metrics to use in these types of studies, but hopping behaviour and time spent in the shelter (if available) could also be used. Finally, based on data in Chapter II and III of this thesis as well as some of the studies reviewed, the variance in some of the behaviours tested was quite large, and the sample sizes were too small to achieve the recommended statistical power of 0.8. An apriori test using effect sizes from literature to optimize the trade-off between type 1 and 2 statistical errors.

In Chapter III, I examined female responses to 11-O-ETIO and its derivatives in tanks without enhancements (no shelter or gravel) (Experiment 1) and in tanks equipped with a PVC shelter and gravel substrate (Experiment 2). Experiment 1 used mixtures of 0.1nM HPLC fractionated RM CW extract. Experiment 2 used similar mixtures of 1μM synthetic analogs with a focus on the effects of 11-O-ETIO-3-s. Despite the issues evident in Chapter III, (sample size, contradictory results to previous studies, low concentration), the experiments do suggest that 11-O-ETIO-3-s is an essential component of reproductive male round goby sexual communication. The goal of this study was to determine which steroid component(s) were important in eliciting female attraction, with the overall objective that this component(s) may one day be used as bait in a traps used to manage invasive round goby populations in the Laurentain Great Lakes. Individually, some of the steroids elicit different responses in females, although not all of them have been tested in a consistent manner. 11-O-ETIO (free) is attractive

to NFs but not RFs (Tierney et al., 2013), 11-O-ETIO-17-s does not elicit an olfactory response in females, but 11-O-ETIO-3-s does (Laframboise & Zielinksi, 2011).  The glucuronated steroids do not appear to be attractive based on our study but further testing is still required.  Based on release rates and previous studies on behavioural responses, 11-O-ETIO-3-s appears to be a good candidate for further testing.  Typically receivers require a mix of pheromones applied at a specific ratio in order to achieve a response similar to that achieved in nature (Sorensen et al., 1998).  Based on the evidence presented in Chapter III, it is possible that 11-O-ETIO-3-s could elicit attraction in females when presented alone which would be highly beneficial for trapping as it is more cost effective to produce a single steroid.  Further studies are required to determine the efficacy of using 11-O-ETIO-3-s alone.

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APPENDIX A: COMPLETE REVIEW TABLE

| Author (year) | Test Tank | | | | Time of year |
|---|---|---|---|---|---|
| | Type | Volume | Temp °C | Enhancements | |
| Tierney et al (2013) See also Kereliuk (2009)- MSc Thesis | rectangle, glass (20 cm) | 5 L | 18 | none | May-Oct (spawning season) |
| Kasurak et al (2012) See also Kasurak (2010) - MSc Thesis | Flume >1m | 96 L | 18 | none | June-August |
| Murphy (1998)- MSc Thesis | rectangle, glass | 100 L | 18 | gravel & PVC shelter | May-August |
| Gammon et al (2005) | rectangle, plexiglass 16 cm long flume | 20 L | 18 | nest shelter, clear top | May-September |
| Yavno & Corkum (2009) | 1 m long flume | 20 L | 18 | nest shelter, clear top | May-August |
| Corkum et al (2008) | Y- flume (1m) | 19 L | 18 | shelter | May-August |
| Yavno & Corkum (2011) | 1m long flume | 2L | 22 | None | May to August |
| Marentette & Corkum (2008) | 1m long flume | 15L | 19 | black shelter clear roof | May-July |

| Author (year) | Time of day | Sample Size (per treatment) | Behavioural Metrics | Time bins |
|---|---|---|---|---|
| Tierney et al (2013) See also Kereliuk (2009)- MSc Thesis | evening | 7 to 15 | Time in inflow (25%) | speed= 3s intervals, time spent = % per min |
| | | | Swimming Activity (speed/hops) | |
| | | | Gill vent. Rate | |
| Kasurak et al (2012) See also Kasurak (2010) - MSc Thesis | NA | 8 | Time spent in upstream box | NA |
| | | | Time in stimulus zone | |
| Murphy (1998)- MSc Thesis | NA | 6 Males and 6 Females | Gape | Continuous |
| | | | Head Lift | |
| | | | Cough | |
| | | | Fanning | |
| | | | Roll Over | |
| | | | Movement | |
| | | | Hop | |
| | | | Nest entry/exit | |
| | | | Gill vent. Rate | every 3rd min |
| | | | Activity | |
| Gammon et al (2005) | NA | 4 to 5 | Time spent in inflow half | |
| | | | Swimming velocity | 1 min every 3 |
| | | | Pathway of movement | min 3-12 |
| Yavno & Corkum (2009) | 9am-7pm | 7 | Time spent in nest | NA |
| Corkum et al (2008) | NA | 8 | Time spent in far half of arms | NA |
| | | | Velocity | |
| | | | Path of movement | |
| Yavno & Corkum (2011) | NA | 12 | Time in upper 3/8ths of flume | NA |
| Marentette & Corkum (2008) | NA | 8 | Time in shelter vs. inflow | per second for 1 min, every three minures |
| | | | Activity level | |
| | | | Head orientation | |

| Author (year) | Observation Method | Acclimation | Odours | Concentration Delivered |
|---|---|---|---|---|
| Tierney et al (2013) See also Kereliuk (2009)- MSc Thesis | Video recording + ethovision | 24h | L-alanine | $10^{-5}$ M |
| | | | Mix Synthetic: 11OE, 11OE- | $10^{-6}$ M |
| | | | Urine | $10^{-5}$ M/$10^{-6}$ M |
| | | | Urine extract | $< 10^{-6}$ M |
| | | | Conj Fractions | $10^{-6}$ M |
| | | | Free Fractions | $10^{-6}$ M |
| | | | Vehicle Blank | NA |
| Kasurak et al (2012) See also Kasurak (2010) - MSc Thesis | Video recording | 5h | Call | NA |
| | | | CW extract | $10^{-5}$ M |
| | | | Call + CW extract | $10^{-5}$ M |
| | | | VB (extracted H2O) | NA |
| Murphy (1998)- MSc Thesis | Manual | 5 days | E1 | $10^{-7}$ M (1mL) |
| | | | E2-3g | $10^{-7}$ M (1mL) |
| | | | ETIO | $10^{-7}$ M (1mL) |
| | | | DHEA-s/DHEA-g | $10^{-7}$ M (1mL) |
| | | | E1, E2-3g, ETIO, DHEA-s | $10^{-7}$ M (100µl ea.) |
| | | | ETIO-g, Andr-s, DHEA-g | $10^{-7}$ M (100µl ea.) |
| | | | Ethanol Control | 1 mL |
| | | | Food (brine shrimp) | 1 g in 100ml |
| | | | L-alanine | $10^{-4}$ M |
| Gammon et al (2005) | Video Recording | 30 min | RM CW | ~$10^{-5}$ M |
| | | | NRM CW | ~$10^{-5}$ M |
| | | | RF CW | ~$10^{-5}$ M |
| | | | NRF CW | ~$10^{-5}$ M |
| | | | Control (dechlor. H2O) | NA |
| Yavno & Corkum (2009) | Video Recording | 1h | RM unrine | 0.2mL (pure urine) |
| | | | NRM urine | 0.2mL (pure urine) |
| | | | male model | NA |
| Corkum et al (2008) | Video Recording | NA | All (free + conjugate) | $10^{-7}$ M |
| | | | ETIO-s, ETIO-g, 11-O-ETIO-s, 11-O-ETIO-g | |
| | | | 11-KT, Ad, ETIO, 11-OE | |
| Yavno & Corkum (2011) | video | 1h | Goby egg washings | NA |
| | | | Trout egg washings | |
| | | | River Water | |
| Marentette & Corkum (2008) | video | 20 min | 1L RM CW | NA |
| | | | 1L NM CW | |
| | | | 1L RF CW | |
| | | | 1L NF CW | |

| Author (year) | Flow Rate | Stimulus Length |
|---|---|---|
| Tierney et al (2013) See also Kereliuk (2009)- MSc Thesis | 5mL/min | 10 min |
| Kasurak et al (2012) See also Kasurak (2010) - MSc Thesis | 2.7 cm/s | 10 min |
| Murphy (1998)- MSc Thesis | none | 34 min |
| Gammon et al (2005) | 6 mL/min | 30 min (used first 15 min) |
| Yavno & Corkum (2009) | 40-45mL/min | 15 min |
| Corkum et al (2008) | 60 mL/min | 20 min (used last 12) |
| Yavno & Corkum (2011) | 25ml/min | 15min |
| Marentette & Corkum (2008) | 30-35mL/min | 20 min (used last 8 mins) |

APPENDIX B: GSI AND TOTAL LENGTH OF FEMALES USED

| 2012 | | | 2013 | | |
|---|---|---|---|---|---|
| Fish ID | GSI (%) | Total Length (cm) | Fish ID | GSI (%) | Total Length (cm) |
| 062712-1 | 12.30 | 7.60 | 060413-1 | >8 | 8.60 |
| 062712-2 | 10.60 | 7.60 | 060413-2 | >8 | 7.60 |
| 062712-4 | >1 | 8.70 | 060413-3 | >8 | 9.10 |
| 062712-3 | 14.20 | 7.20 | 060413-4 | >8 | 7.20 |
| 070412-1 | 8.50 | 7.10 | 062113-1 | 15.30 | 8.50 |
| 070412-2 | 7.70 | 8.40 | 062113-2 | 11.60 | 7.20 |
| 070412-3 | 11.70 | 9.50 | 062613-3 | 14.70 | 10.10 |
| 070412-4 | 5.99 | 7.00 | 062613-4 | 10.70 | 9.40 |
| 070512-1 | 17.00 | 7.50 | 071013-1 | 17.80 | 10.00 |
| 070512-2 | 15.60 | 8.50 | 071013-2 | >8 | 9.10 |
| 070512-3 | 2.50 | 9.70 | 071013-3 | <8 | 18.60 |
| 070512-4 | >1 | 7.00 | 071013-4 | <8 | 18.50 |
| 070612-1 | 2.37 | 9.00 | 071613-1 | 1.35 | 10.00 |
| 070612-2 | 11.70 | 7.30 | 071613-2 | 8.10 | 10.00 |
| 070612-3 | >1 | 6.50 | 071913-3 | 6.24 | 8.40 |
| 070612-4 | >1 | 7.00 | 072613-1 | >8 | 8.30 |
| 071012-1 | 16.00 | 7.50 | 072613-2 | >8 | 9.10 |
| 071012-2 | 2.20 | 8.60 | 072613-3 | 18.00 | 7.40 |
| 071012-3 | >1 | 8.80 | 072613-4 | 13.00 | 8.50 |
| 071012-4 | 7.83 | 8.20 | 073013-1 | 13.00 | 9.00 |
| 071212-1 | 6.80 | 10.10 | 082013-1 | 0.25 | 9.60 |
| 071212-2 | 10.40 | 9.00 | 082013-2 | 1.29 | 8.30 |
| 071212-3 | >1 | 7.90 | 082013-3 | 0.10 | 7.50 |
| 071212-4 | 9.70 | 6.50 | 082013-4 | 0.70 | 7.60 |
| 072812-1 | >1 | 9.20 | 082313-1 | 0.70 | 9.50 |
| 072812-2 | 8.90 | 7.80 | 082313-2 | 0.30 | 9.50 |
| 072812-3 | 5.30 | 9.50 | 082313-3 | 0.10 | 9.50 |
| 072812-4 | >1 | 8.00 | 082313-4 | 1.20 | 9.90 |
| 080812-1 | >1 | n/a | 083013-1 | 1.50 | 9.20 |
| 080812-2 | >1 | n/a | 083013-2 | 12.93 | 9.20 |
| 080812-3 | >1 | n/a | 083013-3 | 0.10 | 7.80 |
| 080812-4 | >1 | n/a | 083013-4 | 1.00 | 9.50 |

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| | |
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