Behavioural responses of female round gobies (*Neogobius melanostomus*) to putative steroidal pheromones

Lynda D. Corkum$^{1,3)}$, **Benjamin Meunier**$^{1)}$, **Michele Moscicki**$^{1)}$, **Barbara S. Zielinski**$^{1)}$ & **Alexander P. Scott**$^{2)}$
(^{1} Department of Biological Sciences, University of Windsor, Windsor, ON, Canada N9B 3P4; ^{2} Weymouth Laboratory, The Centre for Environment, Fisheries, and Aquaculture Science, Weymouth, Dorset DT4 8UB, UK)

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Summary

Although reproductive (RF) and non-reproductive (NRF) female round goby are attracted to washings of conspecific reproductive males and RF, respectively, behavioural responses of females to synthesized steroids has not been studied. We tested attraction and avoidance of RF and NRF to different blends of steroids previously shown to be either produced by reproductive males and/or detectable at a concentration of at least 10^{-8} M by the olfactory epithelium of RF. Three blends of steroids were used, including: etiocholanolone (3α-hydroxy-5β-androstan-17-one), 11-oxo-etiocholanolone (3α-hydroxy-5β-androstane-11,17-dione), androstenedione, 11β-hydroxy-androstenedione, and 11-ketotestosterone ('free' steroid blend); etiocholanolone glucuronide, etiocholanolone sulfate, 11-oxo-etiocholanolone glucuronide and 11-oxo-etiocholanolone sulfate ('conjugated' steroid blend); and all nine steroids together ('total' steroid blend). NRF were attracted to the free steroid blend and avoided the conjugated blend. RF did not reveal any significant bias to the steroid blends, but there was a tendency for RF to prefer conjugated steroids and avoid free steroids. Because there was no significant attraction by RF to particular blends of synthesized steroids tested, other compounds (sex attractants) not yet identified from the male round goby are likely responsible for initiating courtship and/or spawning behaviours in reproductive females.

Keywords: pheromones, round goby, steroid blends.

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[3] Corresponding author's e-mail address: corkum@uwindsor.ca

Introduction

Chemical signals in fishes are important in eliciting reproductive behaviour (Liley & Stacey, 1983). It has been shown that pheromones can be used by individuals to distinguish between reproductive and non-reproductive conspecifics (Partridge et al., 1976), to initiate courtship behaviour (Tavolga, 1956), to increase the concentration of reproductively important hormones and milt production in mature males (Stacey & Sorensen, 1986; Scott et al., 1994) and to synchronize spawning interactions (Kobayashi et al., 2002).

Just as ornaments or elaborate displays are used by individuals to enhance their mating success, odours represent a strong gender-specific signal in sexual selection (Wyatt, 2003). Depending on the species, male or female odours can be used to attract members of the opposite sex. Historically, commercial fishers seeded traps with sexually mature female *Ictalurus punctatus*, to increase catches of mature males (Timms & Kleerekoper, 1972). Adult males also respond to hormonal pheromones released by conspecific females in *Bathygobius soporator* (Tavolga, 1956), *Carassius auratus* (Sorensen & Stacey, 1999) and *Carassius carassius* (Olsén et al., 2006).

Alternatively, pheromones produced by reproductive males attract females to spawning sites in several fishes, including *Oncorhynchus mykiss* (Newcomb & Hartman, 1973), *Danio rerio* (Bloom & Perlmutter, 1977), *Gobius jozo* (Colombo et al., 1980), *Pimephales promelas* (Cole & Smith, 1992) and *Neogobius melanostomus* (Gammon et al., 2005). MacInnis & Corkum (2000) determined that a single sexually mature male round goby (*Neogobius melanostomus*) attracted up to 15 conspecific reproductive females into an artificial nest.

The round goby is a small teleost that invaded North America from the Ponto-Caspian region of Eurasia presumably by ballast water transported in transoceanic freighters (Charlebois et al., 2001). Recently documented nighttime vertical migration by round goby larvae may explain the rapid dispersal of the species (Hensler & Jude, 2007); the round goby has spread to all five Great Lakes within 5 years of its introduction (Charlebois et al., 2001). A broad diet, tolerance to a wide range of environmental factors, aggressive behaviour, male parental care, and multiple spawning periods each year contribute to the success of the round goby (MacInnis & Corkum, 2000).

Round gobies exhibit negative impacts on the environment, by transferring contaminants from the sediments (round gobies feed on Dreissenidae) to the upper levels of the food web, where gobies are preyed upon by sport and commercial fishes (Jude et al., 1995; Morrison et al., 2000). Additionally, round gobies feed on the eggs of native fishes (Steinhart et al., 2004), displace other benthic fishes (Jude et al., 1995; Dubs & Corkum, 1996) and contribute to the bycatch in nets of commercial fishers (Corkum et al., 2004). Accordingly, physical (trapping, barriers), chemical (piscicides) and biological (pheromone strategy; Corkum, 2004; or acoustic devices, Rollo et al., 2007) approaches could be used to control this nuisance species. In October 2005, the Ontario Ministry of Natural Resources and Fisheries and Oceans Canada cooperated to apply rotenone to the Pefferlaw Brook, a tributary of Lake Simcoe with the sole purpose of eliminating round goby from the tributary to prevent its spread into Lake Simcoe, a lake with a multimillion dollar sport fishery (B. Brownson, OMNR, personal communication). Of these techniques, biological controls are advantageous because of the potential of focusing on the target species. The round goby inhabits dark turbid benthic environments and so it is likely that pheromones and/or acoustic signals, rather than visual stimuli, play a vital role in the reproductive success of this species. This notion supports an earlier finding that sex discrimination in the blind goby *Typhlogobius californiensis* (albeit a different subfamily than *Neogobius*) occurs by chemoreception (MacGintie, 1939).

Members of the Gobiidae family respond to water in which a conspecific has been placed. For example, in *Bathygobius soporator*, exposure to water into which an ovulatory female has been placed leads to immediate initiation of courtship behaviour in reproductive males (Tavolga, 1956). When reproductive round goby females are exposed to water into which a reproductive male had been placed, these females swim towards the odour source and remain there (Gammon et al., 2005). Reproductive females (RF) swim significantly faster than non-reproductive females (NRF) towards reproductive male donor water, suggesting that reproductive males (RM) emit a releaser pheromone.

Physiological assays (electro-olfactogram, EOG) by Murphy et al. (2001) showed that the round goby olfactory system did not respond to prostaglandins, but did detect free and conjugated 18-, 19- and 21-carbon steroids, especially 19-carbon steroids in which the A-ring had a 5β-reduced configuration. Using EOG recordings, Zielinski et al. (2003) showed that RF responded significantly more than NRF to solid-phase (octadecylsilane) extracts from reproductive male round goby water. When these extracts were separated on reverse-phase high-performance liquid chromatography (HPLC), RF showed noticeably greater responses than NRF to the fractions that eluted 'early' (between 30 and 40 min; Belanger et al., 2004), suggesting that they were conjugated rather than free steroids (Arbuckle et al., 2005).

Arbuckle et al. (2005) showed that a suite of steroids, both free and conjugated, are synthesized in the testes of sexually mature male round gobies. Several steroids were successfully identified: testosterone (T), 11-ketotestosterone (11-kT); androstenedione (AD); 11β-hydroxy-androst-4-ene-3,17-dione (11β-OH-AD); 3α-hydroxy-5β-androstan-17-one (etiocholanolone, ETIO); 3α-hydroxy-5β-androstane-11,17-dione (11-oxo-ETIO); and 11-oxo-ETIO-s. Two of these steroids, 11-oxo-ETIO and 11-oxo-ETIO-s, had not been previously identified in teleost gonads (Arbuckle et al., 2005).

One of the principle steroids synthesized by the mesorchial glandular mass of the testis of *Gobius jozo* (Colombo et al., 1980) is etiocholanolone glucuronide (ETIO-g). Exposure of female *G. jozo* to ETIO-g also stimulated attraction and egg deposition by ovulated females. Murphy et al. (2001) detected strong EOG responses of adult male and female round gobies to ETIO-g. In contrast, Arbuckle et al. (2005) failed to demonstrate its in vitro biosynthesis by testes of the round goby. However, it was pointed out that, due to technical difficulties, there was a long time lapse ($>$4 h) in that study between testis collection and subsequent in vitro incubation with radioactive steroid precursors and that such delays had already been associated in the literature with a loss of glucuronide formation. Steroids conjugated as sulfates or glucuronides are better excreted in the urine and bile than others due to their higher solubility in water (Colombo et al., 1980).

In the present paper, we conducted a laboratory experiment to determine whether RF and NRF show a behavioural response to mixtures of steroids identified by Arbuckle et al. (2005), along with ETIO-g and 11-oxo-ETIO-g that, despite their apparent absence in testis incubations, were nevertheless shown to be readily detected by the olfactory epithelium of females (Murphy et al., 2001; Belanger, 2003). Because others (e.g., Sorensen et al., 2005) have shown that sexual arousal in fish occurs as a result of a mixture of steroids (but at different times during courtship and spawning), we studied the effect of steroid blends identified from gonads of male round gobies on luring conspecific females. Additionally, testing blends of steroids increased the likelihood of eliciting a behavioural response by a conspecific female and was a more economical approach (vs. individual steroids) given the difficulty in obtaining adequate numbers of reproductive specimens and that a single 'behaviour guiding' steroid in this species is yet unknown. Because other fishes, *Clarias gariepinus* (Resink et al., 1989) and *Gobius jozo* (Colombo et al., 1980) are known to release conjugated steroidal pheromones to attract females, we tested two blends (conjugated and free) of round goby steroids. Accordingly, we grouped the compounds into free (11-kT, Ad, 11β-OH-Ad, ETIO and 11-oxo-ETIO), conjugated (11-oxo-ETIO-s, 11-oxo-ETIO-g, ETIO-s, ETIO-g) and free + conjugated steroids. Each group was tested against an ethanol control to determine if RF and NRF was attracted or deterred from steroid blends. Based on previous studies mentioned above, we hypothesized that only RF would respond to the steroid blends and that the conjugated steroids would be more potent than the free steroids.

Materials and methods

Animals

Round gobies were collected by angling from the Canadian shore of the Detroit River at Windsor, ON (42°20′N, 82°56′W) and the northwestern shore of Lake Erie at Leamington, ON (42°03′N, 82°36′W) during the morning from early May through mid August, the peak reproductive season for this fish (MacInnis & Corkum, 2000). Fish were housed at the University of Windsor Animal Quarters in accordance with the University of Windsor Animal Care Guidelines.

Upon arrival in the laboratory, female round gobies were housed in separate groups (RF and NRF) and kept under constant photoperiod (16 h light : 8 h dark). Aerated holding tanks (38 l) were equipped with flow-through dechlorinated water held at a temperature of 18 ± 2°C throughout the experimental period. Tanks were lined with aquarium gravel and contained PVC tubing for shelter. Gobies were fed Nutrafin® fish flakes three times per week.

Female round gobies were identified by their broad urogenital papilla (Miller, 1984). Reproductive status was confirmed after experimental trials by sacrificing the fish and examining and weighing the ovaries. The mass of the ovaries was expressed as a percentage of total body mass — the gonadosomatic index (GSI) (Table 1). A value of 8% or higher was taken as an indication of RF status because the body cavities of these females were filled with ripe eggs (Gammon et al., 2005). Additionally, the reproductive females had round eggs with a well-defined yolk centre. Total length of the fish was measured, while the fish was lying on its side, to the closest 1 mm (Table 1).

Preparation of steroids

The nine steroids used in this experiment were 11-kT, Ad, 11β-OH-Ad, ETIO, ETIO-s, ETIO-g, 11-oxo-ETIO, 11-oxoETIO-s and 11-oxo-ETIO-g. The steroids were grouped into conjugated steroids (ETIO-s, ETIO-g, 11-oxo-ETIO-s and 11-oxo-ETIO-g), free steroids (11-kT, Ad, 11β-OH-Ad, ETIO and 11-oxo-ETIO), and total (all nine steroids). ETIO was obtained from Sigma-Aldrich Canada (Oakville, ON, Canada); 11-oxo-ETIO-g was donated by A.P. Scott; all other steroids were obtained from Steraloids (Newport, RI, USA).

Since water flow through the naris changes in synchrony with ventilation, the rate of ventilation is expected (and has been shown) to increase in response to odours (Nevitt, 1991; Murphy et al., 2001; Belanger et al., 2006). Because Belanger (2003) showed that round gobies increased ventilation rate to concentrations of steroids at a threshold of 10^{-10} M, we used this concentration in our study. Solutions were prepared by dissolving powdered steroid in ethanol to achieve a stock concentration of 10^{-4} M. This stock was then diluted with distilled water to a concentration of 10^{-7} M. All stock solutions were kept at −20°C. On the morning of a trial, all steroid blends to be used that day were prepared by taking 100 μl of stock from each steroid required for the blend (conjugated, free, or total) and adding these to the same test tube. The corresponding amount of diluted EtOH was added to another test tube. Test tubes were covered in Parafilm® and left in the freezer until needed. At the end of each trial, the final concentration of steroid in the experimental tank was 10^{-10} M.

Laboratory experiments

Laboratory experiments were conducted to determine if females (RF and NRF) respond to blends of synthesized steroids. A Y-shaped tank was used; stem and arms were each 50 cm long and 15 cm high; width (of stem and arms) was 12 cm. Before each trial, the tank was rinsed out with dechlorinated water and drained through a valve in the bottom until empty and then wiped dry. Shelters and dividers used in the tank were also rinsed and dried. The tank was then filled with dechlorinated water (18–20°C) up to 10 cm, corresponding to 19 l. Four Masterflex L/S® pumps were attached to the Y-tank and all pumped at a rate of 60 ml/min. One air stone was located at the end of each arm to facilitate downstream flow.

Each 1-h experimental trial (following the protocol of Gammon et al., 2005) consisted of three 20-min periods: acclimation, control and stimulus. During the acclimation period, a fish was placed in the stem of the tank near the outflow. A solid barrier was placed 21 cm from the back of the tank to prevent the fish from leaving the area. During the 20-min control period, dechlorinated water was pumped into both arms of the tank and the solid barrier was removed, allowing the fish free movement throughout the tank. During the 20-min stimulus period, a randomly assigned steroid blend (free, conjugated, or total) dissolved in ethanol was pumped into one arm of the tank while an equal amount of ethanol carrier was pumped into the other arm. Treatment and control were randomly assigned to each branch for each trial by tossing a coin.

A RF or NRF was randomly selected for each trial between 24 and 48 h after capture and all three treatments (free, conjugated, total) for each female type (RF, NRF) representing one replicate (i.e., 6 fish) were completed before beginning the next set. A new fish was used for each trial; fish were not reused. The following treatments were analysed: RF with 'free' steroid blend ($N = 8$), RF with 'conjugated' steroid blend ($N = 8$), RF with 'total' steroid blend ($N = 8$), NRF with 'free' steroid blend ($N = 8$), NRF with 'conjugated' steroid blend ($N = 9$) and NRF with 'total' steroid blend ($N = 8$).

Two additional experiments were conducted to test the response of RF ($N = 7$) and NRF ($N = 7$) to both the free and conjugated steroid mixtures when pumped at the same time into the Y-tank. The selection of the free or conjugated mixture that was added to a designated arm of the Y-tank was determined by a coin toss. The only difference in the steroids used in the experiments was in the formulation of ETIO-s. Etiocholanolone potassium sulfate was used in the 'treatment' vs. 'control' trials; etiocholanolone sodium sulfate was used in the 'conjugated' vs. 'free' trials.

Behavioural data analysis

Each trial was videotaped using a colour camera (Hitachi VKC-370) positioned above the flume. Trials were simultaneously recorded on DVD. The activity of the fish was analyzed using FishTracker software (Shen, 2005) during the last 12 min of the stimulus periods, ensuring that the odour was well mixed to determine (1) time spent by the female in the far half of each arm, (2) average velocity of the female round goby and (3) path of movement by the female. Dye tests showed that it took 8 min for dye to move through the tank and so only the last 12 min (20 min – 8 min) were used for analysis (preference index).

We adopted the analytical approach of Li et al. (2002) and used a preference index (I) to determine which arm of the Y-tank was preferred by female round goby. For each fish tested, $I = (A_\text{t}/(A_\text{t} + B_\text{t})) - (A_\text{c}/(A_\text{c} + B_\text{c}))$, where B_t and A_t represent time spent searching the treatment arm of the tank before and after the addition of the stimulus, respectively, and B_c and A_c represent the time searching the control arm of the tank. When conjugated (cj) and free (f) steroids were placed in different arms of the same tank, values for A_t and A_c were replaced by A_cj and A_f, respectively, and values for B_t and B_c were replaced by B_cj and B_f, respectively. Results of a 2-tailed t-test (with 7 df) are presented to determine whether the females were attracted or deterred from steroid blends. Data for each case were normally distributed. Separate 2-factor (3 steroid types × 2 reproductive status) ANOVA tests were used to compare the mean swimming velocity of the fish. When results were significant, Student–Newman–Keuls (S–N–K) post-hoc tests were used to determine which groups did not differ from one another. No quantitative analyses were performed on fish pathways.

Results

Results of a one-way ANOVA showed that there was a significant difference in the mean GSI of females used in the study ($F_{7,63} = 31.84$; $p < 0.0001$; Table 1). Although there were no significant differences in mean GSI values among NRF treatments or among RF treatments, mean GSI of RF was significantly larger than that of NRF (Newman–Keuls multiple comparison test, Table 1). There were no significant differences in mean total length (TL)

Table 1. Mean (SE) preference index gonadosomatic index (GSI) and total length (TL) for non-reproductive (NRF) and reproductive (RF) females for free, conjugated, and total steroids vs. control treatments in a Y-tank.

Females	N	GSI (mean ± SE)	TL (mean ± SE)	Treatment	I	t	p
NRF	8	3.27 (0.619)^a	10.1 (0.35)^a	Free	0.273 (0.131)	2.087	0.075
NRF	9	2.93 (0.638)^a	10.0 (0.26)^a	Conjugated	−0.222 (0.216)	1.028	NS
NRF	8	3.90 (0.880)^a	10.2 (0.49)^a	Total	0.322 (0.193)	1.667	NS
NRF^1	7	1.44 (0.492)^a	8.3 (0.235)^b	Conj vs. Free	−0.116 (0.232)	0.501	NS
RF	8	12.81 (1.166)^b	10.2 (0.28)^a	Free	0.150 (0.241)	0.622	NS
RF	8	11.58 (0.950)^b	10.6 (0.54)^a	Conjugated	0.027 (0.172)	0.370	NS
RF	8	12.51 (1.131)^b	10.2 (0.57)^a	Total	0.240 (0.210)	1.140	NS
RF^1	7	11.09 (0.758)^b	9.0 (0.18)^a	Conj vs. Free	0.259 (0.142)	1.827	NS

Values for NRF^1 and RF^1 represent the response by females to conjugated vs. free steroids. Superscripts ‘a’ and ‘b’ indicate that there was no significant difference in GSI values within NRF or within RF. The NRF used in the Conjugated vs. Free test were significantly smaller than females used in all other treatments. Results of a 2-tailed t-test are presented to determine whether females were attracted or deterred from steroid blends. See text for explanation of I. NS, not significant.

among any of the female groups except NRF used in the comparison of conjugated vs. free steroids; these females were smaller than females used in the other groups ($F_{7,63} = 3.29$; $p = 0.005$; Table 1). There was a significant positive relationship between TL and mass of females for both RF ($r^2 = 0.95$, $p < 0.001$) and NRF ($r^2 = 0.95$, $p < 0.001$). Analysis of covariance showed that there was no significant difference in the relationships between the slopes of the lines ($p = 0.130$), but intercepts were significantly different from one another ($p \leqslant 0.001$). The larger mass of the RF compared with NRF for a given length is attributed to gonad development.

Overall, females (NRF and RF pooled) spent significantly more time (mean ± SE) in the far half of the steroid arm (305 ± 42 s) than in the far half of the control arm (88 ± 21 s) during the last 12 min of the stimulus period (ANOVA; $F_{1,72} = 19.80$, $p < 0.001$) for all cases of treatment (free, conjugated and total) vs. control (Figure 1).

There were differences in the responses between female type and chemical stimuli. The NRF showed a slight preference (2-tailed t-test, $t = 2.087$, $p = 0.075$), indicated by increased time spent in the arm that received the free-steroid vs. the control (ethanol carrier) treatment (Table 1, Figure 2).

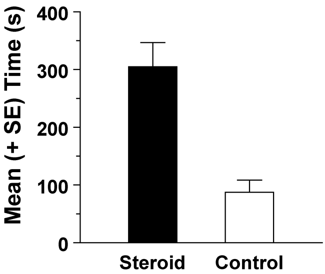

Figure 1. Mean time spent for RF and NRF combined in steroid (black) vs. control (open) arm. There was a significant difference (ANOVA, $F_{1,72} = 19.80$, $p < 0.001$) between the time (mean + SE) RF and NRF combined spent in the steroid arm (305 ± 42 s) vs. the control arm (88 ± 21 s).

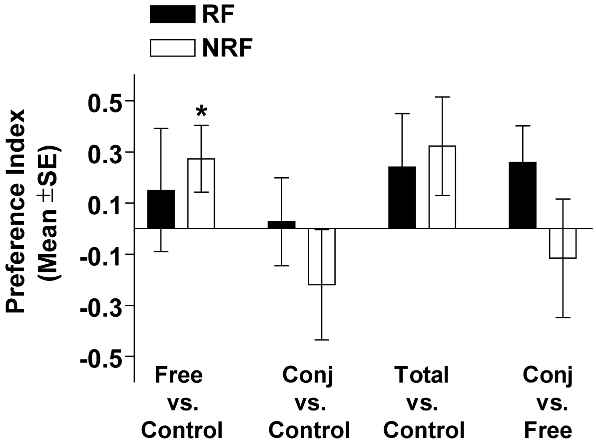

Figure 2. The mean ± SE of a preference index (see text for explanation) to determine which arm of the Y-maze was preferred by female (RF, shaded bars; NRF, open bars) round goby. Positive values represent attraction to free, conjugated (conj) and total steroids when compared with the ethanol carrier control arm or attraction to conjugated steroids when compared with free steroids (conj + free). Negative values represent avoidance. The asterisk represents significant results of a 2-tailed t-test, indicating a difference ($p = 0.075$) between free steroid and control odours.

Although NRF exhibited no significant preference when offered the choice between the other stimuli and the control, there was a trend towards avoidance of the conjugated steroid blend and attraction to total steroids. When NRF were offered a choice between conjugated and free steroid blends, there was a trend to spend more time in the arm with the free steroid

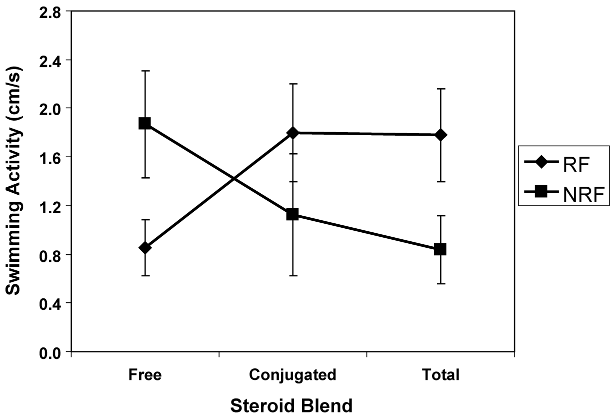

Figure 3. Mean ± SEM swimming activity for RF and NRF exposed to blends of steroids. There was a significant interaction between reproductive status and steroid blend (ANOVA, $F_{2,36} = 4.04$, $p = 0.026$). NRF exposed to free steroids and RF exposed to conjugated and total steroid blends showed greater swimming activities compared to RF exposed to free steroids and NRF exposed to conjugated and total steroid blends.

blend compared to the arm with the steroid blend, but the difference was not significant (Figure 2).

The RF did not reveal a bias to any of the three steroid stimuli vs. control treatments. However, there was a trend towards attraction to total steroids (Figure 2). When RF were offered a choice between conjugated and free steroids, females spent more time in the arm with the conjugated steroid blend compared to the arm with the free steroids, but the difference was not significant (Figure 2).

There were no significant main effects for female swimming velocity during the last 12 min of the stimulus period; however, there was a significant interaction between reproductive status and steroid blend for comparisons between steroid types and the control ($F_{2,36} = 4.04$, $p = 0.026$). NRF swam more (mean ± SE) when exposed to free steroids (1.87 ± 0.44 cm/s) than RF exposed to free steroids (0.85 ± 0.23 cm/s) (Figure 3). However, RF were more active when exposed to both conjugated (1.8 ± 0.40 cm/s) and total (1.8 ± 0.38 cm/s) steroids than NRF exposed to both conjugated (1.1 ± 0.50 cm/s) and total (0.84 ± 0.28 cm/s) steroids (Figure 3).

No quantitative analysis was performed on the fish pathways; however, some general trends were observed. When fish were first added to the tank, there was a brief period during which the fish did not move. During the acclimation period, the fish began to move within the contained area (distance of

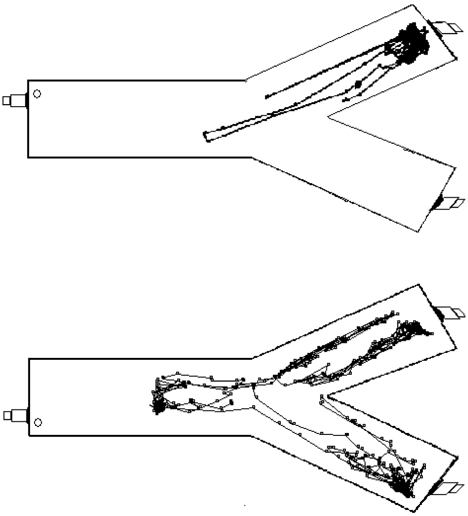

Figure 4. Sample pathways of two trends observed in fish movement. Top panel: RF exposed to free steroids and NRF exposed to conjugated and total steroids showed a pattern of swimming back and forth mainly along the arm containing the steroids. Lower panel: NRF exposed to free steroids and RF exposed to conjugated and total steroids showed a consistent pattern of swimming back and forth along both the steroid and control arms.

21 cm). When the fish moved to the other end of the tank, its body was held at an angle and its snout projected downward touching the tank bottom. The fish moved back and forth with overall progression towards the stimulus. RF and NRF were active within 1 to 4 min of the beginning of the last 12 min of the stimulus period, corresponding with dye tests showing that the steroid should reach the fish within 8 min of the beginning of the stimulus period. The swimming activity of both RF and NRF was similar. Once movement was initiated, a female swam repetitively from side to side along the width and length of the Y-tank. The female either remained in the steroid arm near the steroid source, thus leading to a decrease in swimming activity, or would continue to swim up both treatment and control arms, occasionally pausing in the stem of the Y-maze.

RF exposed to free steroids showed a slight deviation from the above pattern; they swam more along the length of the steroid arm. NRF exposed to conjugated and total steroids also showed this pattern (Figure 4, top panel). Conversely, RF exposed to conjugated and total steroids swam more along the length of the steroid and control (ethanol) arms. This pattern was mirrored by NRF exposed to the free steroid blend (Figure 4, bottom panel).

Discussion

Regardless of reproductive status, females spent more time near synthesized steroids compared with the control (Figure 1), indicating that females are lured to the mix of synthesized steroids that represent compounds that have been isolated from reproductive male (RM) round goby testes. Most promising was the significant attraction of NRF to the free steroid mix of steroids and an avoidance response to the conjugated steroid blend. This behaviour confirmed the findings by Gammon et al. (2005), who showed that not only were RF attracted to RM washings, but that NRF were attracted to washings of RF. Steroids in water from RF round gobies have not yet been analysed, but we anticipate that they contain components the same as, or similar to those present in the free steroid blend (Ad, 11-kT, 11β-OH-Ad, ETIO and 11-oxo-ETIO) and that the ingredient or mixture attracts NRF. If a pheromone control strategy is to be realized as a management tool for the invasive round goby, it would be useful to attract all females, not just reproductive females, to odour traps.

We speculate that inter- and intra-sexual odours might have several functions. In spring, male round gobies are first to move shoreward from the profundal zones of lakes, followed by females (Corkum et al., 2004). The pooled sex attractant released by a dense colony of nesting RM likely lures RF to breeding areas. Shipwrecks, with many cavities and rubble for nests, are nursery areas for round gobies in western Lake Erie (Wickett & Corkum, 1998). Odour trails of RF might guide NRF to the breeding colony (Gammon et al., 2005). Shohet & Watt (2004) showed that female guppies, *Poecilia reticulata*, prefer water containing the odour of sexually mature females over that of control water and this attraction was believed to be involved in shoaling. Because eggs in adult female round gobies hydrate quickly, RF odour may speed up the sexual development of NRF. Finally, NRF may track RF odours because the smell is associated with eggs (food stimuli). Wickett & Corkum (1998) showed that juvenile gobies feed on eggs of nest-holding males when the male was occupied in deterring nest intruders.

Females, regardless of reproductive status, show EOG responses to ETIO and ETIO-g (Murphy et al., 2001). Androgens such as AD and testosterone play a role as substrate for the production of estrogens in female fish (Lin et al., 1991). Also, 11-kT has been found in several female fishes (Matsubara et al., 2005; Lorenzi et al., 2008). Although there are many examples of species in which females produce pheromones to attract males, such as channel catfish *Ictalurus punctatus* (Timms & Kleerekoper, 2001) and signal crayfish *Pacifastacus leniusculus* (Stebbing et al., 2003), this phenomenon does not occur in female round gobies (Marentette & Corkum, 2008).

Because there was no significant attraction by RF to particular blends of synthesized steroids tested, other compounds (sex attractants) not yet identified from the male round goby are likely responsible for initiating courtship and/or spawning behaviours in reproductive females. Our failure to demonstrate a significant preference of RF to either free, conjugated or a total mixed of synthesized steroids was disappointing given the positive response of RF to RM washings reported by Gammon et al. (2005). Thus, it seems likely that (1) the steroid blends are imperfect (concentrations too high or too low or in the wrong proportions); (2) there are steroids in the blend that are not normally released by RM, but are able to initiate a response in RF; and/or (3) there are other compounds released by RM that remain to be identified. In regard to the first two possibilities, we do not yet have enough information on the exact identities (and amounts) of steroids that are released into the water by RM (as opposed to being synthesized by the testes). A compound that cross-reacts in an immunoassay for 11-oxo-ETIO, has the same chromatographic properties and responds to injection of gonadotropin-releasing hormone has been firmly identified in washings of RM round goby (unpublished data). There is also immunoreactive material in the conjugated steroid fraction. However, the conjugated fraction has not yet been firmly identified.

Vrieze & Sorensen (2001) showed that the migratory pheromone released by sea lamprey needs elements of stream water odours to enable the actions of the pheromones. This also may be the case with the round goby; i.e., something may be present in lake water conditioned by RM that is needed to sufficiently activate the steroids to attract RF.

Certainly, if pheromones released by RM are linked to mate attraction, then they should not attract NRF. Colombo et al. (1980) showed that female *G. jozo* had a decrease in their sensitivity to ETIO-g after deposition of eggs. Female round gobies exhibit EOG responses to ETIO-g regardless of reproductive status (Murphy et al., 2001). Belanger (2003) and Jasra et al. (University of Windsor, unpublished data) showed differences in EOG responses between RF and NRF to 11-kT, 11-oxo-ETIO, ETIO-g and 11-oxo-ETIO-g and ETIO-s, but no significant differences between females of different reproductive status for ETIO.

We had anticipated that the response of RF to conjugated steroids would be more potent than their response to free steroids, supporting the findings of Colombo et al. (1980) and Murphy et al. (2001). However, our results show that when given a choice between a conjugated steroid and a control in a Y-maze, there is a neutral response by RF to the odours. When given a choice between conjugated and free steroids, there is a stronger tendency (although not significant) for RF to be attracted to the mix of conjugated steroids. In both cases, the activity levels of the RF are high. Since 65% of wild nests with males in spawning colours lack eggs (Wolfe, 2002), activity may be a measure of courtship behaviour. Time in a nest or near an odour source may indicate spawning behaviour.

RF were equally active, with respect to swimming speed, when exposed to conjugated and total steroid mixtures compared with significantly lower activity levels when RF were exposed to free steroids. This response pattern has also been shown in ripe female *G. jozo* exposed to ETIO-g (Colombo et al., 1980) and ripe female fathead minnows *Pimephales promelas* exposed to RM conditioned water (Cole & Smith, 1992).

The increase in swimming activity may be sustained by the RF searching for the paternal male, i.e., the odour source. This is evident in the pathways shown for RF response to conjugated and total steroids, i.e., the female continually swims up and down the length and width of the steroid arm instead of remaining stationary at the odour source. The RF also swims up and down the length of the control arm, increasing the swimming activity level for these two treatment groups. Swimming in the control arm may occur because the odour eventually leaks from the treatment arm around to the control arm of the tank, as observed in dye tests. Thus, RF detect this overflow of steroid, even at the more dilute concentrations in the control arm.

Flowing water also may affect the response of fish to odours. For example, brown bullheads *Ameiurus nebulosus* are substantially worse at locating an odour source in flow as opposed to no flow (Sherman & Moore, 2001). The implications of this for the present experiment are that the RF may show increased activity to conjugated and total steroids because they are attempting to locate the source of the odour. RF do not show this increased activity to free steroids.

Our next task is to test individual rather than blends of steroids that lure a female to nest-holding male round gobies. Specifically, differences in the ratio of the blends may selectively influence fish in different reproductive states

(Wertheim et al., 2005). Houck et al. (2007) in their study of pheromone communication on the red-legged salamander, *Plethodon shermani*, stress the distinction between sex attractants (signals that lure a conspecific to the general vicinity of the male) and courtship pheromones that function once the mate has been identified and preliminary courtship behaviours are initiated (Houck, 1986).

In the round goby, there are likely several different types of stimuli involved in sex attraction and mating, including vision (nest-holding males in the presence of other conspecifics are black vs. mottled females), vocalizations (non-reproductive round gobies move to speakers emitting a conspecific call, Rollo et al., 2007) and pheromones (Gammon et al., 2005; this study). Recently, we have videotaped spawning of round goby in the laboratory (S. Yavno and B. Meunier, University of Windsor, unpublished data) and all three types of stimuli (vision, sound, pheromones) seem to play an important role in reproductive behaviour.

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