

Reproductive female round gobies (*Neogobius melanostomus*) are attracted to visual male models at a nest rather than to olfactory stimuli in urine of reproductive males

Stan Yavno & Lynda D. Corkum¹⁾

(Department of Biological Sciences, University of Windsor, Windsor, ON,
Canada N9B 3P4)

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Summary

Fish are known to communicate in many ways and commonly use olfactory and visual signals. When round goby (*Neogobius melanostomus*) males become reproductive, they change from mottled grey to black and release sex steroids in their urine. In this study, we conducted a laboratory experiment to determine if reproductive female round gobies were attracted to a combination of olfactory (urine) and visual (silicone models) stimuli, representing reproductive and non-reproductive male round gobies. Females spent significantly more time at a nest with a black reproductive male model compared with a mottled non-reproductive male model. Neither urine type nor the interaction between model type and urine affected the time spent by reproductive females at a nest. Knowledge of the reproductive habits of the round goby may enable researchers to develop a method of species control for this invasive fish by manipulating its breeding habits.

Keywords: fish models, mate attraction, olfaction, urine, vision.

Introduction

Fish respond to stimuli in many ways, but rely predominately on vision and olfaction to reproduce (Liley & Stacey, 1983). These two signalling modalities convey messages between conspecifics to attract mates, initiate

¹⁾ Corresponding author's e-mail address: corkum@uwindsor.ca

courtship and spawn; and, depending on the breeding system, to defend fertilized eggs and offspring. Male nuptial colouration is designed to both attract females and deter male competitors (Kodric-Brown, 1990). Additionally, several species release sexual scents via their urine, which elicit significant behavioural and/or physiological responses in conspecifics (Colombo et al., 1982; Almeida et al., 2005; Appelt & Sorensen, 2007). Sex pheromones play an important role in species recognition, mate recognition and mate assessment (Wyatt, 2003; Johansson & Jones, 2007).

The round goby (Gobiidae: *Neogobius melanostomus*), a bottom-dwelling nuisance fish, entered the Laurentian Great Lakes from Eurasia via ballast water (Jude et al., 1992). The success of the invasive round goby in becoming established in new areas is due in part to its broad diet, repetitive annual spawning, and male parental care (Corkum et al., 2004). This species uses a polygynous mating system in which many reproductive females deposit eggs in the nests of a single male (MacInnis & Corkum, 2000). Parental males are black; whereas non-reproductive males and females are mottled, mimicking colours of bottom substrates (Miller, 1984; Wickett & Corkum, 1998). Washings from reproductive male round gobies initiate a strong behavioural response in reproductive females with observable changes in time spent near the source of the male odour (Gammon et al., 2005). Recently, urine has been shown to be the main excretion route for sex pheromones in the round goby (Kereliuk, 2009). Round gobies pose a threat by feeding on eggs of native fishes (Steinhart et al., 2004), transferring contaminants up the food chain (Jude et al., 1995), out-competing other species (Jude et al., 1995; Dubs & Corkum, 1996) and by contributing to the bycatch in nets of commercial fishers (Corkum et al., 2004). Knowledge of the reproductive habits of the round goby may enable researchers to develop a method of species control by manipulating its breeding habits.

In this study, we tested the relative strengths of visual (models) versus olfactory (urine) stimuli in attracting female round gobies in a laboratory flume. We expected that (1) reproductive females should exhibit a stronger attraction to urine collected from reproductive males than to urine obtained from non-reproductive males; and (2) a reproductive (black) model that represents a parental male should be more attractive to gravid females compared with a non-reproductive (mottled) model, resulting in the movement of the female to a nest.

Materials and methods

Animals

Round gobies were collected by angling along shoreline areas of the Detroit River at Windsor, ON (42°20'N, 82°56'W) and Lake Erie at Leamington, ON (42°03'N, 82°36'W) from May to August (2007, 2008) and May (2009). Because fish captured were not injured and quickly acclimated to holding tanks (feeding immediately and actively swimming), we concluded that angling did not influence subsequent behaviour of the fish.

Round gobies were sexed by the shape of the genital papilla — broad in females and pointed in males (Miller, 1984). Reproductive status was confirmed after experimental trials by sacrificing the fish and examining and weighing the gonads. In the lab, reproductive and non-reproductive males and females were held in separate holding tanks with a flow-through system, air stone, and gravel. Reproductive females were used in experiments within 7 days of capture; urine was obtained from males 24 h after capture. Fish were fed daily with Nutrafin® fish flakes, and held under a 16/8 h light/dark cycle with water temperature $18 \pm 1^\circ\text{C}$. These holding conditions were based on previous studies (e.g., Gammon et al., 2005).

In females, the mass of the ovaries was expressed as a percentage of total body mass, the gonadosomatic index, GSI. A value of 8% or higher was taken as an indication of reproductive status; i.e., the body cavities of these females were filled with ripe eggs (Gammon et al., 2005). The GSI values (mean \pm SE) for all reproductive females used in our experiments were $11.46 \pm 0.061\%$. Additionally, the reproductive females had round eggs with a well defined yolk centre. There was no significant difference ($t_{40} = 1.249$, $p = 0.219$) in mean (SE) GSI of reproductive females collected from the Detroit River ($11.96 \pm 0.57\%$, $N = 21$) and Lake Erie at Leamington ($10.97 \pm 0.54\%$, $N = 21$) nor in the size (total length) of reproductive females ($t_{40} = 0.085$, $p = 0.932$) between the two populations (Detroit River: 9.31 ± 0.27 cm, $N = 21$; Leamington: 9.28 ± 0.29 cm, $N = 21$).

Collection of male urine

To obtain sufficient amounts of urine from males, we initially anaesthetized the males with clove oil, and used dental floss to tie their papillae for 4 h to prevent urination. Urine was extracted from reproductive and non-reproductive male round gobies using a syringe (25 gauge needle); samples

(including dechlorinated control water) were stored at -20°C until needed. In other studies, male round gobies were designated as reproductive if the GSI value was $\geq 1.38\%$ and non-reproductive if the GSI value was $\leq 0.4\%$; i.e., gonads were transparent and miniscule in size (Belanger et al., 2006). In our study, we obtained urine from many males but only selected urine samples for experiments from gonadally developed (reproductive) and gonadally regressed (non-reproductive) males. There was a significant difference ($t_{22} = 16.29$, $p < 0.0001$) between the mean \pm SE GSI values for reproductive ($2.02 \pm 0.194\%$) and non-reproductive ($0.22 \pm 0.037\%$) males from which we obtained urine.

Preparation of models

To prepare the round goby male models, we mixed USG[®] regular dental plaster to create a mould of the gobies. A non-toxic, odourless silicone compound called Oomoo 30[®] (Smooth-on Plastics, Easton, PA, USA) was used to cast the two round goby models. The compound was tinted black while still in the liquid (un-set) phase to represent the reproductive male model. After the removal of the set silicone, the non-reproductive male model was painted with tinted Oomoo 30[®] to create a mottled appearance. The models were designed to represent reproductive and non-reproductive males in both total length, 15 cm, and head width, 3 cm (Figure 1). The mean \pm SE total length (TL) of specimens used for urine extraction was 13.53 ± 0.20 cm (reproductive male) and 12.89 ± 0.24 cm (non-reproductive male).

Laboratory experiment

We used a 2 (model) \times 3 (urine) factorial ANOVA design to test if reproductive female round gobies were attracted to either olfactory or visual stimuli from males. Using both reproductive and non-reproductive male models, we conducted trials with reproductive urine ($N = 7$), non-reproductive urine ($N = 7$) and a control, i.e., dechlorinated water ($N = 7$); i.e., 42 reproductive females were used. All 42 trials (3 urine types \times 2 male models \times 7 replicates) were randomized and fish were used only once. All experiments were conducted between 09:00 and 18:00 h.

A silicone model (either reproductive or non-reproductive male) was placed in a plastic shelter ($16 \times 11.5 \times 5$ cm) with opaque walls and transparent roof at one end of the flow-through flume ($1 \text{ m} \times 30 \text{ cm} \times 30 \text{ cm}$)

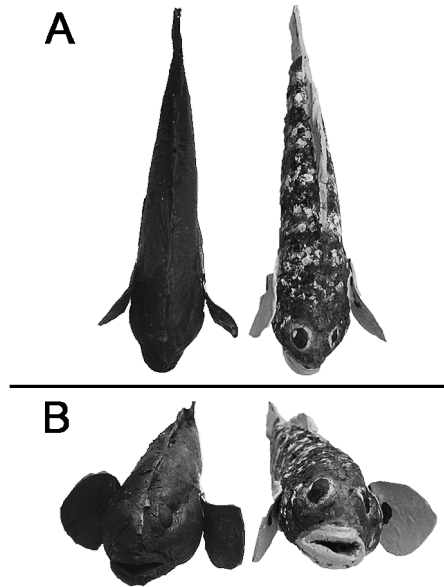


Figure 1. Dorsal (A) and anterior (B) views of reproductive (black) male and non-reproductive mottled male round goby are presented.

containing 20 l of dechlorinated, aerated water (Figure 2). Water flow in and out of the metre-long tank was controlled with a Gilmont® 6.5 mm industrial flow meter (Gilmont Instruments, Racine, WI, USA) and ranged from 40 to 45 ml/min. Valves at the opposite end of the flume were set so that water was removed at the same rate as it entered. A reproductive female was placed in a shelter at one end of the flume. After a 1-h acclimation period, a designated urine type was injected into the tube entering the flume behind the shelter containing the model at the opposite end of the tank (Figure 2). The two shelters contained small holes along their respective rear walls that allowed water to pass through, preventing the build up of stagnant water. Water temperature in the flume was maintained at $18 \pm 1^\circ\text{C}$, a temperature at which round gobies are known to reproduce (Charlebois et al., 1997).

Trials were conducted under fluorescent lights, consisting of a 1-h acclimation period (where dechlorinated water flowed into the flume), and a 15-min stimulus period in which 0.2 ml (the maximum obtained) of urine from reproductive or non-reproductive males or dechlorinated water (control) entered the flume. An opaque gate located 50 cm from the odour source, which kept the females from visual contact with the model, was lifted imme-

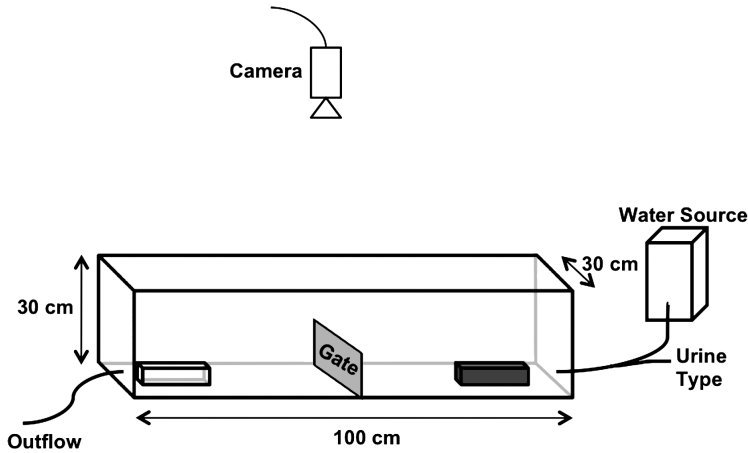


Figure 2. Sketch of flume indicates the relative positions of the holding shelter for the reproductive female and the shaded shelter for the male model. Urine type (reproductive, non-reproductive or control water) was added at the end of the nest where male models were positioned.

diately following an injection of urine or control water into the flume. The final concentration of urine and control water in the flume was 0.00001%.

The criterion for female mate choice was the total time spent in the area at and along the sides or back of the nest. Because our earlier studies on spawning behaviour with live parental males (Meunier et al., 2009), showed that females and males appeared to evaluate the status of each other before the female entered the nest, we selected time spent at the nest occupied by a model as the most appropriate surrogate for mate choice. 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).

Results

Observations obtained from the video images of the reproductive females showed that females moved from one end of the flume to the other by swimming along the bottom and along the side walls of the tank. The dependent variable, time spent at the nest, included the total time at the nest entrance and between the front of the nest and the back of the flume where the nest was positioned. Results of the ANOVA test showed that model type (visual

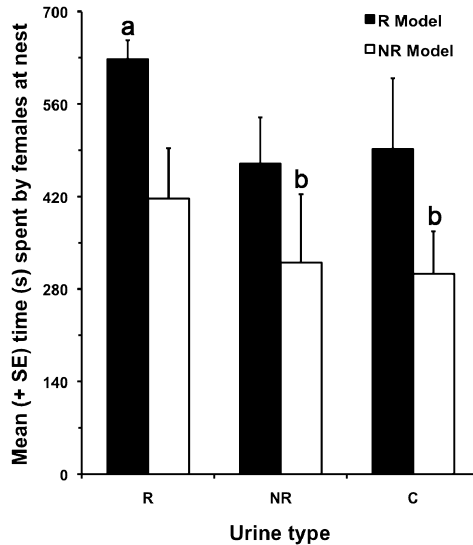


Figure 3. Mean + SE time (s) spent by reproductive females at the nest with the fish model at the far end of the flume. Black bars represent cases with reproductive (black) males; open bars represent non-reproductive mottled males. Urine was obtained from reproductive males (R), non-reproductive males (NR) and control (dechlorinated water). The letters 'a' and 'b' represent significant differences in responses by reproductive females at the nest.

signal) had a significant effect on the length of time females spent at the shelter (nest) at the opposite end of the flume ($F_{1,41} = 7.957$; $p = 0.008$). In contrast, neither urine type ($F_{2,41} = 1.677$; $p = 0.201$) nor the interaction term of model \times urine type ($F_{2,41} = 0.0753$; $p = 0.928$) had any effect on the time spent by the female at the nest. Overall, reproductive females spent the most time at a nest when it was occupied by a parental (black) male model, regardless of the chemical stimuli (urine from either reproductive or non-reproductive male or control water; Figure 3). Results of Duncan's post-hoc test showed a significant difference in time spent by reproductive females at the nest between treatments with the reproductive black male model with urine from reproductive males (628 ± 29 s) and the non-reproductive mottled model with urine from non-reproductive males (320 ± 104 s), $p = 0.017$. Also, there was a significant difference between the time that reproductive females spent at the nest with a black male model with reproductive male urine and the non-reproductive male mottled model with control water (303 ± 64 s), $p = 0.014$. There was no correlation between the GSI values of the reproductive females and time spent at the nest ($r = -0.179$, $p = 0.256$) nor

between female size (total length) and time spent at the nest ($r = 0.150$, $p = 0.342$).

Discussion

This study showed that the round goby model type (a visual signal) was more effective than urine type (an olfactory signal) in attracting conspecific reproductive females to a nest in a laboratory flume. Specifically, reproductive females spent more time at a nest when it was occupied by a black round goby model than a mottled one. Not all black round gobies are reproductive, but reproductive parental, nest-holding males have black nuptial colouration (MacInnis & Corkum, 2000; Marentette & Corkum, 2008; Marentette et al., 2009). Parental males are black throughout the breeding season from early May until the end of the summer. Sexual selection tends to favour conspicuous colouration; i.e., in our study, a black or contrasting colour if the male leaves the nest, whereas a mottled pattern favours crypsis with bottom substrates (cf., Endler, 1991).

Marentette et al. (2009) present morphological evidence, supporting the existence of male alternative reproductive tactics in the round goby. The parental dark male morph with secondary sexual traits have larger investment in accessory glands and elevated 11-ketotestosterone levels compared with the parasitic light morph that invests more in testes mass and sperm volume (Marentette et al., 2009). Black parental male morphs defend nests during courtship, spawning and development of gametes, unlike the lighter mottled morph that may sneak fertilizations or non-reproductive mottled males that may temporarily occupy shelters (Meunier et al., 2009; Corkum, personal observations).

The mean time spent by a reproductive female at a nest with a reproductive male model was longer in the presence of reproductive than non-reproductive male or control urine, but differences were not significant. Why was the response of the reproductive females to a nest not significantly enhanced in the presence of urine from reproductive males? Previously, Arbuckle et al. (2005) identified a suite of steroids that are synthesized in the testes of sexually mature male round gobies as well as the presence of steroid producing cells in the testes. Recently, Katare (University of Windsor, unpublished data) reported an unknown sulphated conjugate of 11-oxo-etiocholanolone in

round goby male urine. Thus, at least one of the steroids synthesized in the testes is released into the environment via urine. In other studies, we have shown that reproductive females spent more time in the far half of a flow-through tank when washings from reproductive rather than non-reproductive male round gobies were introduced (Gammon et al., 2005), but not when offered a choice of blended synthesized steroids known to occur in the testes of reproductive males (Corkum et al., 2008). Both studies (Gammon et al., 2005; Corkum et al., 2008) conducted under the same environmental conditions (clear water, same temperature) as ours, lacked males or fish models. Interestingly, Kereliuk et al. (2009) recently showed that reproductive female round gobies were attracted to high-performance liquid chromatography fractions of conditioned water (which includes urine) from gonadotropin releasing hormone (GnRH)-injected reproductive males; GnRH is known to increase the release of steroids. The lack of a significant response by reproductive females to male urine in our study may be explained because the males were not treated with GnRH, there was variation in steroid concentrations among reproductive males and/or because the key steroid was not present in sufficient concentrations to initiate a response. Moreover, male stimuli (vision and odour) may result in differential responses by reproductive females given their distance to a nest. We showed that the presence of a visual signal (i.e., male nuptial colouration) is attractive to reproductive females when they are near a nest.

Time spent at the nest by females is assumed to be a good predictor of mating preference (Meunier et al., 2009). Colouration has been shown to influence mate choice in several fishes, including threespined sticklebacks, *Gasterosteus aculeatus* (Baube et al., 1995); guppies, *Poecilia reticulata* (Houde & Endler, 1990); tailspot wrasse, *Hailchoeres melanurus* (Kuwamura et al., 2000); bluegills, *Lepomis macrochirus* (Cogliati, 2009) and others. In contrast to vibrant colours of other fishes, cavity nesting parental males such as mottled sculpins, *Cottus bairdii*; upland bullies, *Gobiomorphus breviceps*; fathead minnows, *Pimephales promelas* and round gobies are typically black (Page & Burr, 1991; McDowall, 2001), presumably to blend in with dark interior of the nest to avoid predation. A nest-holding male protrudes its head from the cavity and briefly exits the nest (Corkum, personal observation), enabling the male to be visible to approaching females.

In the present study, females swam to stimuli by moving along the bottom of the flume and at the junction of the floor and walls of the flume. Such

thigmotactic behaviour is typical of many bottom-dwelling species (i.e., sea lamprey, crayfish) and is advantageous in avoiding predators (Alberstadt et al., 1995; Vrieze & Sorensen, 2001). Round gobies lack a swim bladder and although they are able to enter the water column briefly and 'flit' from one spot to another, all but the early larval stages are benthic (Hensler & Jude, 2007).

Animals have an array of signalling modalities (acoustic, hydrodynamic, pheromonal, visual); however, the main sense organ used depends on the medium in which the signal is transmitted. Reproductive females may process multiple signals when approaching nest-holding parental males and signal strength of a given stimulus may vary with distance from the nest. Once the female is near the nest, nest entry decisions may be determined by colour (as shown in this study), and sounds (Rollo et al., 2007). Also, behavioural displays (pectoral or tail fin fanning) by the male could be detected by the lateral line of females (Meunier et al., 2009). Owing to the parental investment provided by nest-holding round gobies, mutual mate choice that is known to occur in other resource-based mating systems (Kraak & Bakker, 1998) also may occur in this species. Courtship behavioural displays and responses between males and females need to be explored more fully to understand the reproductive habits of this species.

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