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Reproductive Behaviour and Urinary Signals in the Round Goby Neogobiud Melanostomus

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REPRODUCTIVE BEHAVIOUR AND URINARY SIGNALS IN THE ROUND GOBY *NEOGOBIUS MELANOSTOMUS*

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DECLARATION OF CO-AUTHORSHIP / PREVIOUS PUBLICATION

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows: This thesis also incorporates the outcome of a joint research undertaken in collaboration with Stan Yavno under the supervision of Professor Lynda Corkum. The collaboration is covered in Chapter 2 of the thesis. In all cases, the key ideas, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through the data collection and analysis process.
I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis. I certify that, with the above qualification, this thesis, and the research to which it refers, is the product of my own work.

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This thesis includes 1 original paper that have been previously submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status
Chapter 2	First observation of round goby spawning and nest guarding in the laboratory	Submitted

I certify that the above material describes work completed during my registration as graduate student at the University of Windsor.

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ABSTRACT

In studying chemical communication, it is important to characterize how olfactory signals are released and dispersed by the producer before investigating how signals are interpreted by the receiver. In the present study, I used dye injections and a particles image velocimetry technique to characterize the release and dispersion of urine signals by male round gobies *Neogobius melanostomus*. I found that male round gobies release urine signals passively and do not modulate their urination activity in the presence of reproductive females. Additionally, males use repeated tail flippings to generate currents that disperse olfactory compounds in the environment and enhance the detection of this coumpounds by females. Thus, males can advertise their reproductiveness without leaving the nest. Ultimately, the characterization of round goby pheromonal communication will improve our understanding of the role of chemical signals in animals and will be an important asset for the control of the invasive round goby in the Great Lakes.

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CHAPTER 1: GENERAL INTRODUCTION

1. Pheromones and sexual selection

The term pheromone was first proposed by Peter Karlson and Martin Lüsher in 1959. They defined pheromones as “substances which are secreted to the outside by an individual and received by a second individual of the same species, in which they release a specific reaction, for example, a definite behaviour or a developmental process”. A more recent definition of pheromones is ‘odour or mixture of odours released by a 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). Species use chemicals signals in a variety of biological contexts such as feeding, navigating toward a distant source, warning conspecifics against predators, fighting or mating.

Darwin (1871) first recognized the particular importance of olfactory signals in the process of sexual selection. Since then, sex pheromones have been reported in the vast majority of organisms, ranging from insects to mammals (Wyatt, 2003; Johansson and Jones, 2007). Like visual and acoustic signals, sex pheromone signals play an important role in species recognition, mate recognition and mate assessment (Wyatt, 2003; Johansson and Jones, 2007).

2. Functions of sex pheromones

From an evolutionary point of view, two main processes have driven the evolution of sex pheromones (Wyatt, 2003; Johansson and Jones, 2007). On one hand, sex pheromones involved in species and mate recognition have evolved to minimize the cost of searching for, courting and mating with non-suitable partners (Cardé and Baker, 1984; Svensson, 1996). For instance, in mammals, female receptiveness is often advertised by the release of sex pheromones (Johnston, 1983; Marchlewska-Koj et al., 2001). Discrimination of reproductive status from a distance profits males by minimizing the cost of searching for mates, and also profits females by reducing the harassment from males outside the reproductive season (Johansson and Jones, 2007). To convey accurate information about species, sex and reproductive status of an individual, species and mate recognition pheromones must be highly divergent across species, they must be sex-specific and show little variation among individuals (Wyatt, 2003; Johansson and Jones, 2007).

On the other hand, sex pheromones involved in mate assessment have evolved from the benefit accrued of choosing a higher quality mate (Johansson and Jones, 2007). Reproducing with superior mates translate into higher offspring fitness. Therefore, advertising mate quality results in higher reproductive success. To honestly advertise the relative quality of an individual, mate assessment pheromones must vary greatly between individuals and be costly to the producer (Grafen, 1990).

A sex pheromone signal can have more than one function (Johansson and Jones, 2007). For instance, in the cockroach *Nauphoeta cinerea*, the male

pheromone advertises the status of an individual in the male hierarchy, attracts females and is used by females to assess male quality (Moore and Moore, 1999).

3. Sex pheromones in fish

Signals of different nature can be used by species to communicate at different spatial scales. For instance, coral reef fish larvae use sound cues to orientate and navigate toward reefs located kilometers away (Leis et al. 1996, Stobutzki and Bellwood 1998, Tolimieri et al. 2000, Atema et al, 2002) while olfactory cues influence their settlement at smaller spatial scale (Sweatman 1988, Elliott et al. 1995, Arvedlund and Nielsen 1996). In other species, olfactory signals are used to communicate at large spatial scale as in the example of salmonids homing (Dittman et al., 1996). In water, the exchange of visual information and vocal signals can be hampered by turbidity, low light conditions or background noise caused by waves (Columbo et al., 1982). Thus, many fish species have evolved communicatory systems based on the release of pheromones in the water (Sorensen and Stacey, 2006). Contrary to sound and visual signals, chemical signals have the advantage of being easily carried over a great distance by water currents and can go around physical barriers. (Thornhill and Alcock, 1983).

Sex pheromones in fish can be produced by specific glands or arise from changes in body chemistry (Stacey, 2003; Sorensen and Stacey, 2006; Johanssen and Jones, 2007). Specifically, water-borne steroids, prostaglandins

and their metabolites have been shown to induce reproductive behavioural and physiological responses in many fish taxa including common carp, goldfish, catfishes, salmonids, and gobies (Stacey, 2003). Functions of sex pheromones in fish include attracting mates to a nesting site, courting, synchronizing spawning and stimulating gamete production (Sorensen and Stacey, 2004).

The best known fish pheromonal system is the one of the goldfish *Carassius auratus*. In this species, pre-ovulatory females release steroidal pheromones in their urine, which stimulate milt production and sperm motility in males (Sorensen and Stacey, 2002). During ovulation, female goldfish also release prostaglandins in their urine which then induce males into spawning (Sorensen and Stacey, 2002). Interestingly, female goldfish actively advertise their spawning readiness by controlling where and when they release urine and hence any constituent pheromones (Appelt and Sorensen, 2007). A similar behaviour is observed in male Mozambique tilapia *Oreochromis mossambicus*, which increase urination activity in the presence of pre-spawning females but not in the presence of post-spawning females (Miranda et al. 2005). In both of these species, it was shown that individuals modulate their release of pheromone-laden urine according to olfactory signals previously released by the other sex (Miranda et al. 2005; Appelt and Sorensen, 2007).

4. Dispersion of olfactory signals

Contrary to sound and light, pheromones excreted in the water (or in the air) are not “effectively instantaneous” because potent molecules need to be

transported from the producer to the receiver olfactory organs (Wyatt, 2003). The transport of pheromonal signals is affected by factors such as ambient flow and release rate (Atema, 1996; Webster and Weissburg, 2001). Thus, to characterize fish (and other aquatic species) pheromonal systems, it is important to have a comprehensive view of the flow fields surrounding producers and receivers and of the behavioural mechanisms associated with the release of olfactory compounds (Webster and Weissburg, 2001).

Because pheromones actively released and dispersed will have a greater chance of reaching a targeted receptor than pheromones passively leaked into the water, some aquatic species generate their own pheromone excretory currents (Atema, 1996). For instance, male lobsters use pleopod fanning at the entrance of the nesting cavity to disperse pheromones into the environment and advertise their reproductive status to females (Atema, 1986). Additionally, controlled release and active dispersion confer a specific structure to the pheromone dispersal plume. Spatial and temporal information within pheromone dispersal plumes (i.e. pheromone concentration, concentration gradient, flow direction) can be exploited by the receiver to locate and navigate toward the originator of the olfactory signal. Such behaviour is referred as chemically mediated guidance (reviewed in Weissburg, 2000; Zimmer and Butman, 2000).

5. Sex pheromones and control of aquatic pest species

The study of chemically mediated guidance presents a growing interest for the control of aquatic pests (Sorensen and Stacey, 2004, Corkum & Belanger,

2007). In fact, sex pheromone attractants could be used to increase the efficiency of the current trapping techniques employed in pest management programs (Sorensen and Stacey, 2004). The species- and sex-specificity of sex pheromones could enable the targeting of reproductive individuals of a particular pest species and remove their reproductive contribution from the population recruitment (Sorensen and Stacey, 2004). A similar technique is already used with great success for the control of pest insects (Wyatt, 2003). A recent study, showed that female sea lamprey, a fish invader of the Laurentian Great Lakes, can successfully be attracted to traps baited with male mating pheromone 3kPZS (7α, 12α, 24-trihydroxy-5α -cholan-3-one 24-sulfate) (Johnson et al., 2009).

6. The round goby

The round goby *Neogobius melanostomus* is a small benthic fish of the Gobiidae family, which invaded the Laurentian Great Lakes in the 1990's (Jude et al., 1992). Round gobies were accidentally introduced in North America by contamination of ballast water of ships coming from the Ponto-caspian region in Eastern Europe, their natural range (Charlebois et al., 2001). Since then, the species has rapidly spread to the all five Great Lakes (Charlebois et al., 2001). The quick success of round goby in the North-American watersheds is due, in part, to its well adapted reproductive biology (MacInnis and Corkum, 2000). In this species, reproductive males occupy a nest –usually a cavity under a rock– to which they attract reproductive females for spawning. After spawning, males aggressively guard the eggs against predators until hatching. A single male can

guard eggs from up 15 females at a time (MacInnis and Corkum, 2000). Because round gobies spawn in shallow, often turbid waters and males are concealed within spawning cavities, finding a mate can be challenging for the members of this species. It was shown that nesting males produce pheromones that attract gravid females to their nesting cavities (Arbuckle et al., 2005; Gammon et al., 2005).

Evidence of sex pheromone attractants in the round goby suggest that a pest management strategy based on pheromone baited traps could be developed for the control of this invader in the Great Lakes basin. In this perspective, research is ongoing to indentify the active compound(s) and characterize the odor environment associated with male round goby sex pheromones.

7. Objectives of this study

The objectives of the present study were threefold: 1) To analyse the behavioural context in which male round gobies release sex pheromones. 2) To characterize the release patterns of pheromone-laden urine by male round gobies. 3) To identify and quantify factors that could affect the transport of round goby male sex pheromones.

By getting round gobies to spawn under laboratory conditions, we provided the first detailed description of round goby reproduction. We observed that, during spawning, the round goby male behaviour is a subtle balance between attracting females to the nest for mating, defending the nest against

intruders and egg predators and caring for the eggs. We also identified a particular fanning behaviour that likely promotes the dispersion of male pheromones. In a second behavioural assay we determined that reproductive male round gobies do not modulate the release of pheromone-laden urine in the presence of reproductive females. This suggests that male round gobies likely release sex pheromones spontaneously and not in response to a female stimuli. Finally, in a third study, we defined and quantified fanning currents generated by nesting male round gobies. We determined that fanning behaviour in this species, in addition to other functions, likely evolved to promote the dispersion of pheromonal messages by nesting males.

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CHAPTER 2: FIRST OBSERVATION OF ROUND GOBY SPAWNING AND NEST GUARDING IN THE LABORATORY

1. INTRODUCTION

Since it was first reported in 1990 (Jude *et al.* 1992), the round goby (*Neogobius melanostomus*) has been a prolific invader in the Laurentian Great Lakes (Charlebois *et al.* 2001). Reasons for the proliferation of the round goby include its broad diet and availability of molluscan prey (adults eat mainly dreissenids), aggressiveness, high fecundity, multiple spawning (up to six times per year), and male parental care (Corkum et al. 2004). .

Previous field studies have provided evidence for nest defense and egg fanning (Wickett and Corkum 1998) but there has been no direct observation of the patterns of spawning behaviour by the round goby. In this study, we present the first reported account of spawning by the round goby in the laboratory and describe agonistic vocalizations, egg care and nest defense by the parental male. We also provide a review of common nesting behaviours described in other species within the Gobiidae. Understanding the reproductive habits of the round goby could enable researchers to control the spread of this invasive species by manipulating its reproductive success.

2. METHODS

In August 2007, round gobies were collected by angling along the Canadian shore of the Detroit River at Windsor, ON, and brought to the Animal

Care Facility at the University of Windsor. Ten mature fish (3 males and 7 females) were placed into a 90 L tank lined with aquarium gravel and filled with dechlorinated, aerated water; rectangular opaque PVC shelters (16 cm x 11.5 cm x 5 cm) served as nests. Fish were fed daily with Nutrafin flakes and kept under constant photoperiod (16L:8D) at 20°C. At this time, none of the fish showed reproductive characters as described in Miller (1984).

Spawning was induced by changing environmental factors. At first, photoperiod was lowered from 16L:8D to 8L:16D, water temperature from 20°C to 10°C and food supply was restricted to simulate “winter conditions”. After 3 weeks, artificial “spring conditions” were gradually restored (water temperature (20°C), light exposure (16L:8D), daily food supply). Within a few days, one male started displaying typical reproductive traits as black coloration and territoriality (Miller, 1984). During the following weeks, two to three females developed swollen abdomens. Spawning was observed on October 14th, 3 weeks after the beginning of spring conditions. The same protocol was repeated with success in January 2008 (spawning in March 2008), in March 2008 (spawning in May 2008) and November 2008 (spawning in January 2009) using different fish. Spawning typically occurred 3 to 6 weeks after the beginning of artificial spring conditions.

Once the round goby developed secondary sexual characters, male-female interactions were monitored every 2 hours (from 9:00am to 12:00pm). Video recording was started once spawning behaviour was visually confirmed. In October 2007 and May 2008, spawning occurred at night. We were unable to observe the egg deposition but video recording started within 2 hours after the

female had left the nest. At this time, we observed a single male guarding in a nest with eggs deposited on the ceiling. We observed the male fertilizing the eggs, fanning the eggs and guarding the nest. Because the male repeatedly opened and closed its mouth while positioned at the nest entrance, we placed a hydrophone (Interocean Systems Inc, model 902) in the tank 30 cm from the nest entrance to record potential vocalizations.

In March 2008, spawning occurred in the afternoon. This time, we were able to video record egg deposition on the ceiling of the nest by the female and the parental care by the male during the days following the eggs deposition. We video recorded spawning behaviour in October and March using a colour video camera (Hitachi VKC-370) and a DVD recorder (SONY RDR-GX330). In January 2009, we used a new recording device (HDD SONY recorder) enabling us to document, without interruption, the courtship, spawning and parental care of the round goby (spawning occurred at night).

After analysising the digital images from four spawning events (Oct 07, Mar 08, May 08, Jan 09), we were able to describe the main phases of round goby reproduction (mate attraction, egg deposition and parental care) as well as quantify spawning activity, egg fanning, and aggressive displays against intruders. Spawning behaviour recordings may be viewed on the website: www.uwindsor.ca/goby.

3. RESULTS AND DISCUSSION

Despite our success in documenting the first round goby spawning in a laboratory, none of our males completed their brood cycle. We did not observe egg hatching. In some cases, eggs were consumed by round gobies that successfully entered the nest after spawning despite the male's vigilance in nest guarding. In other cases, the parental male terminated their brood cycle by eating the eggs within its nest.

3.1. Spawning Behaviour

During the observed spawning bout, only the male and a female were present inside the nest for the entire recording. Despite the presence of other round gobies in the tank, no intruders entered the nest during the spawning event.

When spawning, the male and the female alternately flipped over (inverted) to deposit their gametes onto the ceiling of the nest. Eggs and sperm were released through the urogenital papilla (Fig 1a, 1b) which was erected at about 45 degrees from the body to make contact with the nest ceiling. Female inversions were almost twice as frequent as male inversions; and, each female inversion lasted four times longer than male inversions. For the March 2008 spawning event, we quantified spawning behaviour over a 30-minute observation period. The female inverted 39 times, while the male inverted 21 times. The male inverted in the nest less than once per minute, for approximately 5 seconds. The female inverted once or twice per minute—a frequency commonly observed in females of the monogamous goby (*Valenciennea longipinnis*) (Takegaki and

Nakazono 1999) —for a duration of about 20 seconds. As in other *Neogobius* species, the female we observed deposited eggs on the ceiling of the nest (Biro 1971, Grabowska 2005); the male divided his time between fertilizing the eggs and guarding the entrance of the nest during the spawning period.

Whenever the female deposited eggs, she performed small but rapid undulating upside down movements on the ceiling, pressing her papilla against the roof of the nest. The male made similar undulating movements when fertilizing the eggs. During periods in which fish were not inverted, both the male and female would rest in the center of the nest. After spawning, the female appeared to depart of her own accord and the male continued to guard the nest.

Observations of round goby spawning events in the Detroit River (MacInnis and Corkum 2000) and western Lake Erie (Wickett and Corkum 1998) revealed that fertilized eggs were deposited on all surfaces of the interior of a nest. Perhaps initial egg deposition begins on the ceiling of the nest and subsequent egg deposition by other females fills the remaining available surfaces. Eggs of round goby are deposited in a single layer (MacInnis and Corkum 2000).

3.1. Parental Care

3.1.1. Vocalization and other agonistic behaviours

Vocalization by reproductive round goby males have been previously identified (Protasov *et al.* 1965, Rollo *et al.* 2007). However, the function of these calls and the manner in which calls are produced remain unclear. During our

laboratory investigations, video and audio recordings showed that nest-guarding males produced vocal pulses by a series of rapid opening and closing of the mouth, reminiscent of a dog barking – see Ladich and Myrberg (2006) for review on sound production mechanisms in fish.

Vocalization started after egg deposition and continued for 2 to 3 days after the female had left the nest. A spectral analysis of the audio recordings revealed the low frequency (1 Hz – 400 Hz) profile of these “barking calls” (Fig. 2). There were variations within and among individuals in the amplitude of these calls and the number of times that they were repeated. However, episodes of intense vocalization had a constant period of 0.4 – 0.6 seconds. Vocalization episodes by the parental male were always associated with the approach of an intruder at the entrance of the nest and typically resulted in the quick retreat by the intruder. Whenever the intruder remained, it was attacked by the parental male. In these cases, the parental male left the nest for a few seconds to chase (and sometimes bite) the intruder.

Similar vocalizations by nesting male round gobies were previously recorded by Rollo et al. (2007) and referred as “pulse series” calls. However the behavioural context in which male round gobies produce these calls remained unclear. Our laboratory observations in the round goby reveal the agonistic nature of these calls. Their function is clearly to discourage intruders from entering the nest. Agonistic vocalizations during territorial defense were previously identified in eight others species of the Gobiidae (reviewed in Amorin and Neves 2008).

Aggressive behaviour also involved various visual displays. In addition to their darkened body and swollen cheeks, threatening nest-guarding males raised their pectoral and dorsal fins upon the approach of an intruder, possibly to increase their body size and better block the access to their nest. When immobile at the nest entrance, one male kept its mouth open continuously, revealing white teeth and gums against a black mouth cavity. This hostile display was also described in the river bullhead, *Cottus gobio* (Morris 1954) and in cichlid fish, *Tilapia natalensis* (Baerends and Baerends van Roon 1950).

On several occasions, we observed intruders entering the nest to feed on the eggs despite the vigilance of the guarding male. However, we did not observe any intruding males, sneaking into the nest to fertilize eggs. Although there is evidence for the presence of sneakers in the round goby (Marentette and Corkum 2008), behavioural confirmation is lacking.

In summary, we identified three levels of agonistic behavior displayed by the parental male in response to intruders. Initially, the male passively blocks the entrance of the nest with his head and pectoral fins. At the next level, the male initiates vocalizations and erects its pectoral and dorsal fins in response to the intruder. At the highest aggression level, the male leaves his nest to physically confront the intruder.

3.1.2. Fanning and Egg Care

Nest fanning is a common behaviour in many species with parental care e.g., three-spined stickleback *Gasterosteus aculeatus* (Reebs *et al.* 1984), river

bullhead *Cottus gobius* (Morris 1954), cichlid fish *Oreochromis niloticus* (Baerends and Baerends van Roon 1950), bluegill sunfish *Lepomis macrochirus* (Coleman and Fisher 1991), and in several species of Gobiidae (Table 1). Ventilation is necessary to prevent sediment build-up inside the nest and to supply eggs with a sufficient oxygen flow (especially for cavity spawners (Gibson 1993)). It has been shown to significantly increase egg survivorship (Östlund and Ahnesjö 1998).

During our observations, nest guarding male round gobies ventilated their nesting cavity using their pectoral and caudal fins (Fig. 1c). Interestingly, we observed that fanning activity started before the egg deposition. For instance, in the January 2009 spawning event, a reproductive male started fanning his nest 10 days prior to the first egg deposition.

In each spawning event, fanning reached a peak activity within a few hours after egg deposition, slowly decreased over the following 48 h and eventually stopped (Fig. 3). Interestingly, these observations differ from fanning behaviour in other gobiids. In the sand goby *Pomatoschistus minutus* (Lindström and Wennström 1994, Järvi-Laturi *et al.* 2008), freshwater goby *Padogobius martensii* (Toricelli *et al.* 1984) and two-spotted goby *Gobiusculus flavescens* (Skolbekken and Utne-Palm, 2001), fanning activity is positively correlated with eggs age and thus progressively increases throughout the brood cycle.

Several reasons could explain differences in fanning behaviour between our observations in *Neogobius melanostomus* and other gobiids, including clutch size, phylogeny, trade-offs with other activities and oxygen concentrations.

Previous studies showed a negative association between fanning and oxygen concentration in gobiids (Torricelli *et al.* 1984, Jones and Reynolds 1999a, Takegaki and Nakazono 1999, Maruyama *et al.* 2008). Because our flow-through tank ensured a constant high dissolved oxygen level in the water (75% saturated or more) our males could have limited fanning expenditures to reallocate their efforts to nest defence (Fig. 4). This idea is supported by Lissåker and Kvarnemo (2006), who showed that because time and energy are limited resources, guarding males face a constant trade-off between nest ventilation and nest guarding. Alternatively, it is also possible that fanning patterns vary across genera. Unfortunately, species that are most phylogenetically related to the round goby, the monkey goby *Neogobius fluvialitis* and the Caspian goby *Neogobius caspius* (Neilson and Stepien 2006) also lack a detailed description of their spawning and egg caring behaviour so that comparisons among these three closely related species are not possible. Finally, others have reported a positive association between fanning activity and clutch size (Lindström and Wennström 1994, Karino and Arai 2006). During our laboratory investigation, only a single female deposited eggs. Since a round goby male can guard eggs from up to 15 females in nature (MacInnis and Corkum 2000), a small clutch size could explain the decrease of fanning activity that we observed.

Other egg care behaviours that we observed were nest excavation and cleaning (Fig. 1d) and egg inspection (Fig. 1e). During nest excavation, the guarding male picked up gravel in its mouth and spat it out of the nest, creating a small mound at the nest entrance. Lissåker and Kvarnemo (2006) reported that

egg guarding sand gobies decreased their nest opening by piling sand at the nest entrance when egg predators were introduced. A small nest opening may also aid in nest concealment (Jones and Reynolds 1999b). However, Svennson and Kvarnemo (2007) found no support that nest size opening was related to female mating preference, nest concealment or that nest size opening served as a physical defence against sneaker males in sand gobies.

During the egg care process, round goby males were often seen lifting their head up to the eggs to “sniff” them and sometimes nibble them (Fig. 1e). This behaviour likely corresponds to an inspection from the male trying to spot unhealthy or dead eggs (Jones and Reynolds 1999c), preventing the spread of diseases in the clutch.

3.2. Filial Cannibalism and Termination of Care

Round gobies successfully spawned in the laboratory, yet none of our males completed their brood cycle and no egg hatching was observed in our tank. In some cases, eggs were consumed by nest intruders. In other cases, males terminated their brood cycle by cannibalising their eggs (only 3 to 5 days after egg deposition). Filial cannibalism is common in many species with parental care and occurs when the cost of care exceeds the benefit (Lissåker and Kvarnemo 2006). Small clutches have a low reproductive value for nest-guarding males (Sargent 1992) and thus have often been associated with filial cannibalism (Sargent 1992, Manica 2002, Lissåker *et al*. 2003, Karino and Arai 2006). During our observations, 150 to 680 eggs were deposited by 1 to 2 females. Since male

round gobies can guard eggs from up to 15 females (MacInnis and Corkum 2000), a small clutch size most likely explains the high rate of filial cannibalism and the lack of hatching success in our study. Because parental care represents an important energetic cost that can compromise the survival of future broods, a male may discontinue care if the benefits are too low. Lissåker and Kvarnemo (2006) proposed that there must be a minimal clutch size for which males decide to discontinue their care. Our observation of round goby spawning showed that males would not complete their brood cycle when eggs came from a single female with few eggs.

4. CONCLUSIONS

In this first description of the round goby reproduction in the laboratory, we showed that the round goby shares many spawning habits with other members of the Gobiidae (Table 1). These spawning habits explain, in part, the success of this invasive species in the Laurentian Great Lakes. Nesting males invest tremendous energy into parental care which has been directly associated with successful establishment of invasive fish species (Drake, 2007). Males use a combination of agonistic vocalizations and visual displays to dissuade intruders from entering their nest. We identified three levels of aggressive response to the approach of intruders. Gradual aggressive responses help males to conserve their energy and to minimize the risks involved in physical confrontations with other individuals (injuries or eggs left without surveillance). Males consistently alternate nest defence and egg care activities. Egg care activities were mostly

dominated by nest ventilation. By repeated movement of their pectoral and caudal fins, males create a constant flow of water inside the nest which provides the eggs with sufficient oxygen and disperses metabolic wastes. We observed that fanning activity can vary throughout the brood-cycle and is likely dependant on oxygen level and clutch size. Interestingly, we observed fanning activity before egg deposition. This suggests that fanning behaviour by male round gobies might play a role during the mate attraction process. The association of fanning and courtship has been previously documented in other species such as the sand goby (Pampoulie et al. 2003) and the three-spined stickleback *Gasterosteus aculeatus* (Sevenster, 1961). It was found that male sand gobies increase their fanning efforts in the presence of potential mates (Pampoulie et al. 2003). In the three-spined stickleback, males perform courtship fanning even in the absence of eggs in their nest (Sevenster, 1961).

We also observed that male round gobies can terminate their care and cannibalize their own brood if the energetic cost of parental care outweighs the reproductive value of the brood (as in the example of a small brood). Sargeant (1992) described this filial cannibalism as a highly adaptive reproduction strategy which allows males to invest their energy into future broods of higher reproductive value.

Finally the present study showed that it is possible to induce round goby reproduction in the laboratory outside of the reproductive season. This finding will have important implications in current and future studies that investigate mechanisms involved in mate attraction within the round goby. Ultimately, the

manipulation of round goby reproductive success could enable researchers to control the spread of this species into new areas.

TABLE 1. Summary of spawning behaviours within the Gobiidae. Species names are listed in alphabetical order, and confirmed behaviours present in males of each species are signified by a checkmark.

Scientific Name	Sole Parental Care	Inversion	Vocalization	Filial Cannibalism	Fanning	Reference
Amblygobius phalaena	✓	✓			✓	Takegaki 2000
Gobius cruentatus			✓			Sebastianutto et al., 2008
Gobiusculus flavescens	✓	✓		✓	✓	Skolbekken and Utne-Palm, 2001
Knipowitschia punctatissima	✓	✓	✓		✓	Lugli et al., 1995
Neogobius fluviatilis	✓	✓				Biro 1971
Neogobius gymnotrachelus	✓	✓				Grabowska 2005
Neogobius melanostomus	✓	✓	✓	✓	✓	Rollo et al., 2007; Wickett & Corkum 1998; Miller 1984
Padogobius martensii	✓	✓	✓		✓	Lugli et al., 1995; Torricelli & Romani, 1986
Pomatoschistus minutus	✓	✓		✓	✓	Jarvi-Laturi et al., 2008; Lindström & Wennström, 1994; Lindström & Hellström 1993; Lissåker et al. 2003
Pomatoschistus pictus			✓			Amorim & Neves, 2007; Amorim & Neves, 2008
Priolepis nocturna	✓	✓			✓	Wittenrich et al., 2007
Rhinogobius brunneus	✓	✓		✓	✓	Takahashi & Kahda, 2004; Suk and Choe, 2002
Valenciennea longipinnis	✓	✓			✓	Takegaki & Nakazono, 2000; Takegaki & Nakazono 1999
Zosterisessor ophiocephalus	✓	✓	✓		✓	Ota et al., 1996; Mazzoldi et al., 2000; Malavasi et al., 2008; Scaggiante et al., 1999

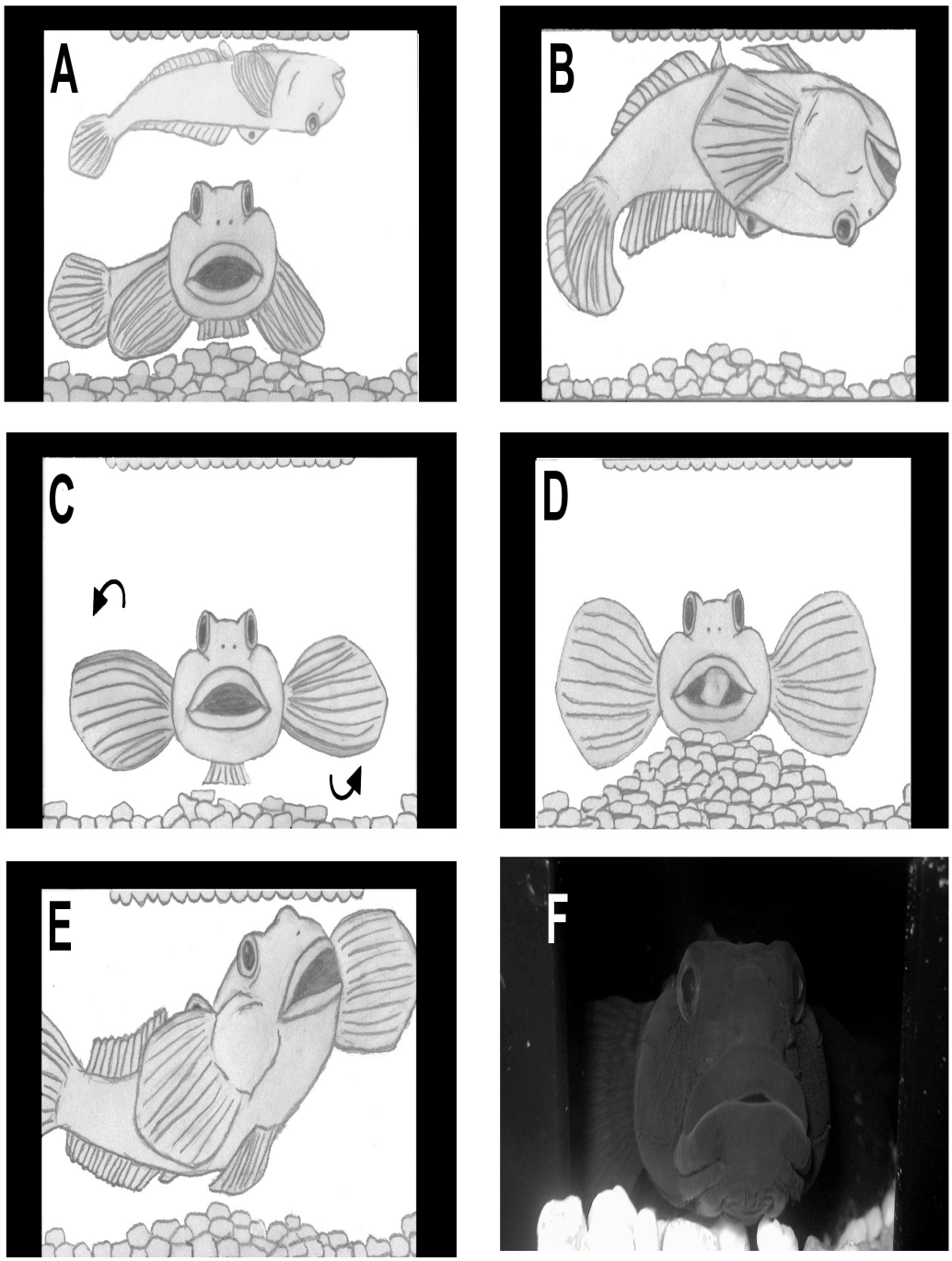

FIG 2.1. Example of spawning and nest-guarding behaviours observed in the round goby: A. Egg deposition by the female, B. Egg fertilization by the male, C. Nest fanning by the male, D. Egg inspection by the male, E. Nest excavation by the male, and F. Photograph of nest-guarding male. Although the nest-guarding male is black (as in F), the drawings depict the male in a lighter shade so that details of the fish may be seen.

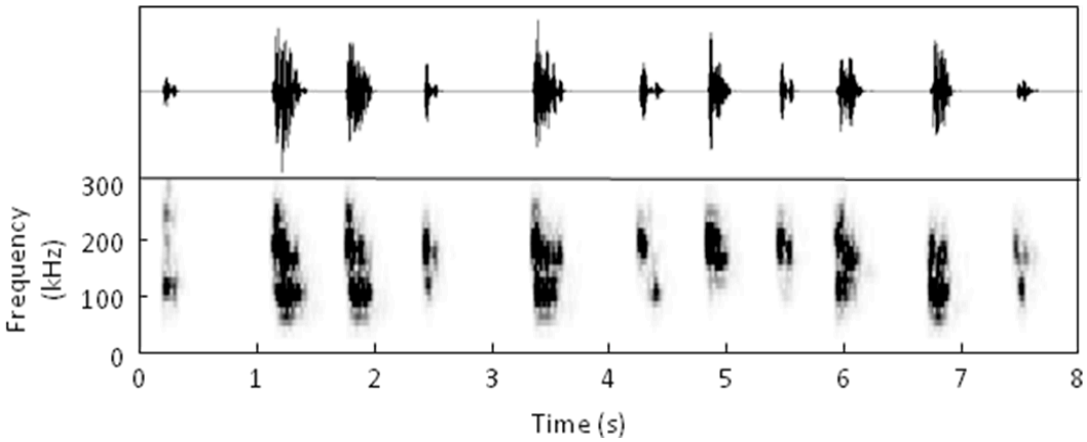

FIG 2. Sound spectrogram depicting an example of an agonistic vocalization produced by a male round goby during nest defence (“barking call”).

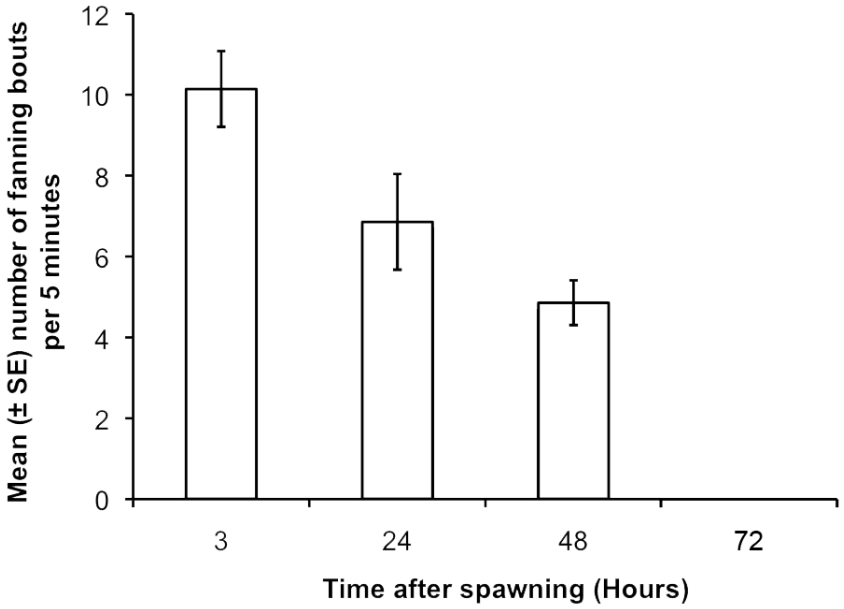

FIG 3. Mean (± SE) number of fanning bouts per 5 minutes observed in a nest-guarding male at 3, 24, 48 and 72 h after spawning.

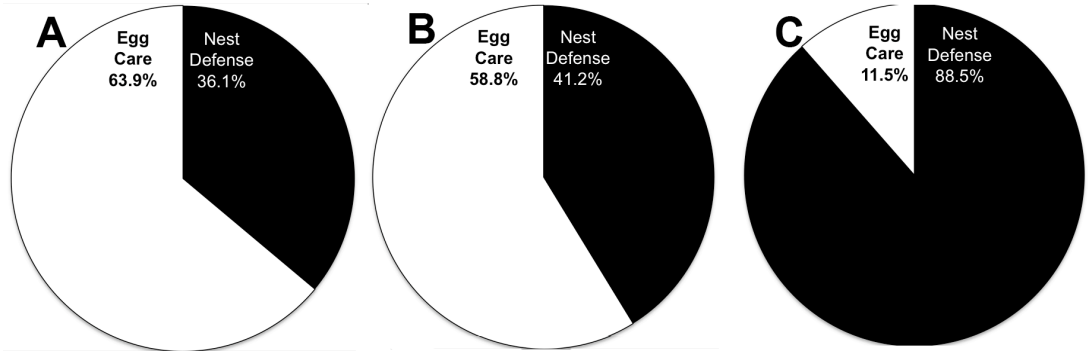

FIG 4. Percentage of time allocated between egg care and nest defense observed in a nest guarding male round goby at A. 3 hours post-spawning, B. 24 hours post-spawning and C. 48 hours post-spawning.

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CHAPTER 3: ARE URINE PULSES PASSIVE CUES OR SPECIALIZED OLFACTORY SIGNALS IN NESTING MALE ROUND GOBY, NEOGOBIUS MELANOSTOMUS?

1. INTRODUCTION

Chemical signalling in fish has been linked to many activities, including predator avoidance and alarm cues (Chivers and Smith, 1998; Wisenden 2003), gender recognition (Liley, 1982), kin recognition (Olsén et al., 1999), aggregations prior to reproduction (Taborsky, 1998), and spawning (Stacey and Sorensen, 2002; Sorensen and Stacey 2004;). Chemical communication between sexes of a given species often consists of individuals of one sex releasing odours to attract a receptive mate (Corkum and Belanger, 2007). For example, sex pheromones produced by reproductive males attract females to spawning sites in several fishes, including *Oncorhynchus mykiss* (Newcomb and Hartman, 1973), *Danio rerio* (Bloom and Perlmutter, 1977), *Gobius jozo* (Colombo et al., 1980), *Pimephales promelas* (Cole and Smith, 1992) and *Neogobius melanostomus* (Gammon et al., 2005). Typically, fish release pheromones through their urine, faeces, gills, skin and sperm (Sorensen et al., 1998). Among these, urine seems to be particularly important for the release of sex pheromones. Several species release sexual scents via their urine, which elicit significant behavioural and/or physiological responses in conspecifics

(Colombo et al., 1982; Liley, 1982; Dmitrieva et al., 1988, Miranda et al. 2005; Yambe et al. 2006; Appelt and Sorensen, 2007).

The success of the small, bottom-dwelling round goby, *Neogobius melanostomus,* as an invader in the Laurentian Great Lakes is, in part, due to its colonial breeding habit and parental care, resulting in high reproductive success (MacInnis and Corkum, 2000). Like other gobiids (Reese, 1964), reproductive males (RM) of the round goby are territorial and occupy nests to which reproductive females (RF) are attracted to spawn. The nest typically consists of a concealed cavity under a stone with a single opening (Charlebois et al., 2001). Round gobies spawn repeatedly throughout spring and summer; and, up to 15 females may deposit eggs in the nest of a single male (MacInnis and Corkum, 2000). Courtship and mate assessment in the round goby, as in other nest-guarding species, are particularly interesting for the study of animal communication as they involve the intimate exchange of visual, vocal and chemical signals between males and females (Millers, 1984; Charlebois et al., 2001; Corkum et al., 2008; Meunier et al., 2009). Chemical signaling is important in synchronizing spawning behaviour in the round goby because the fish often dwells in turbid waters where visual signals are inefficient. Specifically, reproductive female round gobies are attracted by odours from nesting males (Gammon et al., 2005). Behavioural assays showed that odours from reproductive male round gobies (RM) evoke a strong behavioural attraction in preovulatory RF, inducing observable changes in time spent near the male

odour, increased swimming velocities, and directed movement toward the male odour source (Gammon et al., 2005).

Odour from reproductive round goby males is composed, in part, of a suite of putative steroidal pheromones synthesized by specialized cells in the testis (Arbuckle et al., 2005). These active compounds are released into the water (unpublished data) and elicit strong responses from the RF olfactory system (Gammon et al., 2005; Corkum et al., 2008). Although the production of sex pheromones by RM testis appears to be specialized, it is unknown whether RM round gobies can actively control the release of these compounds in the water. For instance, in male Mozambique tilapia *Oreochromis mossambicus* (Miranda *et al.*, 2005) and in female goldfish *Carassius auratus* (Appelt and Sorensen, 2007), fishes increase the release of pheromone-laden urine to actively advertize social dominance and/or spawning readiness to potential mates.

Ultimately, determining the active or passive nature of a signal in a communication dyad comes to resolving which sex signals first. In fact, signal modulation indicates the response of the producer to an earlier signal. For instance in the Mozambique tilapia, pre-ovulatory females release olfactory cues in the water to which males respond by actively increasing the release of pheromone-laden urine (Miranda et al., 2005). Similarly, in the round goby, Murphy et al. (2001) and Belanger et al. (2006) found that RM respond to gonadal extracts and putative pheromones, estrone, from RF, indicating that RM can recognize potential mates based on olfactory cues produced by the RF.

Based on these findings, we hypothesised that RM round gobies control their release of urinary pheromone to actively signal their spawning readiness to potential mates. Hence increasing their reproductive success. If so, RM should significantly increase their urine release (in volume and/or frequency) in the presence of RF to actively signal their spawning readiness to potential mates. In contrast, RM should not modulate their urination activity in the presence of a non-reproductive female (NRF). In the case that RM cannot discriminate between RF and NRF, we expect to observe no significant difference in RM urination activity in the presence of a RF and in the presence of a NRF.

Additionally, we did not expect urine release by non-reproductive males (NRM) to change in the presence or absence of gravid females. We also investigated the importance of urine as a route of excretion for sex pheromone in the round goby by comparing the concentrations of putative steroidal pheromone in urine and washings from RM.

2. METHODS

2.1 Experimental animals

Round gobies were collected twice weekly by angling on the Canadian shore of the Detroit River at Windsor, ON (42°20´N, 82°56´W) and on the northwest shore of Lake Erie at Leamington Harbour, ON (42°03´N, 82°36´W). Fish collection coincided with the reproduction season of round gobies, which takes place between early May and late July, in 2007 and 2008. After collection,

the fish were brought to the laboratory at the University of Windsor, sorted by sex and placed into 95 L communal tanks. Each tank was equipped with flow-through dechlorinated water, aquarium gravel and artificial nests (PVC 16 cm x 11.5 cm x 5 cm). Reproductive males were separated from the other fish and placed into individual 20 L tanks with 1 to 3 reproductive females. The reproductive males were isolated to avoid aggression as well as to maintain and stimulate the males' reproductive behaviour until the day of the trial. Individual tanks were equipped with the same features as the communal tanks. All fish were fed daily with Tetramin Flake Food and kept under a constant photoperiod of 16L:8D at 20±2°C. Individuals were used in an experiment within 7 days of their capture.

At first, sex and reproductive status were established based on morphological characteristics. Females have a blunt urogenital papilla and their abdomen becomes swollen when reproductive, whereas males have a pointed urogenital papilla and become black with swollen cheecks when reproductive (Miller 1984). Because spawning readiness was a critical factor in the experiment, reproductive status was confirmed after each trial by dissecting the fish and thoroughly inspecting its gonads. We assumed that a female was reproductive (RF) when her gonado-stomatic index (GSI= gonad weight x 100 / total body weight) was greater than 7% and/or her ovaries weighted more than 1 g and were fully vascularized with large bright yellow eggs. Additionally, spawning readiness was assessed by applying gentle pressure on the fish's abdomen which, with ripe females, results in the eggs dripping out through the urogenital papilla. Female individuals with gonad weight less than 0.5 g and with

small grayish eggs that did not drip out when applying pressure to the abdomen were considered non-reproductive (NRF). Reproductive male (RM) readiness was confirmed when their GSI exceeded 1.3% and/or their gonad weight was greater than 0.5 g. Testes had to be fully vascularized, opaque and release sperm when gently pressed. To create a clear contrast between the two male reproductive categories (RM and NRM) only juvenile individuals where used in the NRM group. The NRM had small body weight (< 20 g) and immature testes (<0.05 g).

2.2 Excretion of putative steroidal pheromone in RM urine

To determine the importance of the urinary route for the excretion of sex pheromones in the round goby, we compared the concentrations of 11-oxo-ETIO metabolite found in the urine of reproductive males and the concentration 11-oxo-ETIO metabolite measured in water in which reproductive males have been held (washings). Test fish were injected with a solution of gonadotropin-releasing hormone (GnRH) (20μg/Kg in 0.9%saline) to stimulate the production of testicular steroid (Arbuckle et al. 2003). To collect urine samples, we used dental floss to tie urogenital papilla of RM (N=3). The urine, which pooled in the fish bladder, was collected after 4 h using a syringe (see methods in Yavno and Corkum, 2009). Fish washings were collected (N=20) over a period of 16 h during which individuals were held in aerated jars filled with 1 L of dechlorinated water. RM urine and washings were examined for the presence of 11-oxo-ETIOs using an Enzyme-linked immunosorbent assay (ELISA).

2.3 Effect of fluorescein injection

Prior to investigating the urination activity of round goby using fluorescein-injected individuals, we tested whether round goby biology and behaviour is affected by the injection of fluorescein dye. The two following tests were conducted on individuals captured and kept in the same conditions described in 2.1.

2.3. 1 Toxicity

We tested whether fluorescein has a toxic effect on the round goby, by injecting three males of different body sizes (8.6 g, 27.7 g and 61.4 g) with 100 μL of fluorescein sodium solution (1 mg/mL in 0.9% saline). To be conservative, the quantity of fluorescein injected for this test was twice as large as the quantity injected for the urination behaviour experiments. After injection, the three males were then placed into separate 40 L tanks filled with dechlorinated water and equipped with aquarium gravel, shelter and aeration. In each tank, we placed 2 other males (NRM) and 2 females (NRF) to interact with the injected males. Fish were fed every two days with Nutrafin food flakes. During one month we regularly monitored weight, general health, social behaviour, and feeding behaviour of injected and non-injected individuals to determine if the fluorescein had an adverse effect on their biology and behaviour.

2.3.2 Olfactory response

We used an electro-olfactogram method (EOG) adapted from Belanger et al. (2004) to investigate the olfactory response of round goby to fluorescein. The presence of an olfactory potent dye in the experimental tank could disturb the behaviour of test individuals and adversely affect the results of our experiment. We used three stimuli solutions of fluorescein sodium: $C_1=10^{-4}$g/L; $C_2=10^{-3}$g/L; $C_4=10^{-1}$g/L. Stimuli were introduced in the olfactory system through the posterior nostril and the EOG response was recorded differentially using glass electrodes. The recording electrode was placed on the surface of the olfactory mucosa through the posterior nostril while the reference electrode was placed on the fish skin. Differential EOG responses to the three solutions of fluorescein were recorded and compared to responses to a standard solution of alanine (10^{-5}M); three females were tested.

2.4 Male's urination behaviour

2.4.1 Male urination in response to conspecific females

In this experiment, male round goby urination behaviour was investigated in isolation and in the presence of a female. To visualize the release of urine by male round gobies, individuals were injected with fluorescein, an innocuous dye, which fluoresces under UV light. Males were anesthetized by immersion in a light solution of MS-222 (10 mg in 1 L of water) and injected intramuscularly in the dorsal region (between head and dorsal fine) with 50 μL of fluorescein sodium solution (1 mg/mL in 0.9% saline). Subsequently, the injected fish were placed into the experimental tank to acclimatize and allow time for fluorescein to be

excreted in the urine. The experimental tank was a 40 L aquarium filled with dechlorinated, aerated water and a rectangular, U-shaped PVC shelter set on the bottom. Experiments were conducted in low-light conditions (2 ultra-violet “black-light” lamps) so that the fluorescein could be observed in the excreted urine. The tank was placed on a glass-top table and video recordings were made from underneath. This setup enabled the urogenital papilla, located on the ventral surface of the fish, to be seen when the male rested on the bottom of the tank.

A trial started once fish began to release fluorescent urine (45 to 60 min after dye injection). During each trial, a reproductive (RF) and a non-reproductive female (NRF) was sequentially introduced in the experimental tank and allowed to interact with the injected male for 30 min. Each female treatment period (RF and NRF) was preceded by a 30-min isolation period, which served as a control (CtrlNRF and CtrlRF). The order of the stimulus treatments was randomized. Fish (males and females) were used only once. Each trial was video-recorded using a colour video camera (Hitachi VKC-370) and a DVD recorder (SONY RDR-GX330).

2.4.2 Male urination in response to conspecific males

Round goby reproductive males may release urine signals in the presence of any fish, not just female conspecifics. Thus, urine signals may be released during social interactions, not just sexual interractions. To test the response of male urination in the presence of male conspecifics,an experiment was conducted during summer 2008 by Lisa Isabella-Valenzi

(BSc. Thesis). The protocol was similar to the one described above but reproductive and non-reproductive males were introduced in the tanks as stimuli instead of females. Only urination frequency of the resident males was investigated; the volume of urine released was not quantified.

2.4.3 Data analyses

Video-recordings of the trials were examined to determine the frequency at which males urinate and the duration of each urination event (UE). Males typically urinate in two distinctive ways such that males either release a weak stream of urine passively or expel urine with a force as a potent burst. We used a correction factor (1 or 2) to describe these two types of urine discharge (1, weak stream, 2, burst) and then applied this factor to the duration of each UE to obtain an objective estimate of the volume of urine released at each UE. After transformation (Sqrt (UE frequency +1) and Log10 (UE volume +1.1)) to ensure normality and homoscedasticity, data were analyzed using ANOVA (for randomized block design) and *a priori* orthogonal comparison (Statistica, Statsoft, 1998). For each data set (Frequency and Volume), we performed five comparisons (Table 1) to test the following postulates:

1- Males (NRM and RM) increase urination in the presence of a female (regardless of her reproductive status) because they use urinary pheromones as intraspecific signals (planned comparison #1). If both RM and NRM significantly increase their urination activity in the presence of a female

(regardless of her reproductive status) then we proved that urine signals are not produced in a reproductive context. In this case, I anticipate that RM and NRM will increase their urination activity in the presence of another male too.

2- Only RM increase their urination in the presence of a female because they use urinary pheromones as sexual signals. (planned comparison #2 and 3)

3- Only RM increase their urination in the presence of RF to actively signal their spawning readiness to potential mates. (planned comparison #4 and 5)

Variation in the urination frequency of RM in the presence of male conspecifics was tested using a two-way randomized block ANOVA followed by a *post-hoc* test.

TABLE 1: Results of ANOVA and *a priori* orthogonal contrasts for the difference in urination frequency and volume of urination released by male round gobies (RM, NRM) in isolation (CtrlRF, CtrlNRF) and in the presence of a female (RF, NRF).

Planned comparison	Males		Treatment				Number of UE	Volume of urine
#	RM	NRM	CtrlNRF	NRF	CtrlRF	RF	p=	p=
1	1	1	-1	1	-1	1	0.0058	0.0062
2	1	0	-1	1	-1	1	0.1135	0.0223
3	0	1	-1	1	-1	1	0.0218	0.0914
4	1	0	0	-1	0	1	0.3977	0.2051
5	0	1	0	-1	0	1	0.0983	0.2445

3. RESULTS

3.1 Reproductive status and morphological traits of males and females

In males, the mean GSI± SE of RM (2.21± 0.24%; N=31) was significantly different from NRM (0.09± 0.02%; N=20) (t_{49}=7.22, $p < 0.001$). Additionally, RM mean body weight (BW±SE), 28.91 ± 2.52 g, and total length (TL± SE), 127.00 ± 3.97 mm, were significantly higher than in NRM (BW:14.05± 0.74g; t_{49}=4.65, $p < 0.001$; TL:104.30± 1.79 mm; t_{49}=4.39, $p < 0.001$).

In females, RF mean GSI (11.77 ± 0.55%; N = 51) was also significantly higher (t_{100} =17.87, $p < 0.001$) than NRF (1.49 ± 0.17%; N=51). However, there was no significant difference in mean body weight (t_{100}=-0.67, $p = 0.50$) or in total length (t_{100}=1.21, p=0.23) between the two reproductive states. Thus, only reproductive status differed between treatments. RF had a mean body weight of 10.26 ± 0.59 g and mean total length of 88.14± 0.240 mm, whereas NRF had a mean body weight of 9.73 ± 0.51 g and a mean total length of 92.25 ± 0.240 mm.

3.2 Concentration of putative pheromone in the urine

The analyses of fish washings collected over 16 h showed that the total excretion rate of putative pheromonal steroid 11-oxo-ETIO conjugates by GnRH injected RM round goby ranged between 3-36 ηg/h. A similar excretion rate (7-29 ηg/h) of 11-oxo-ETIO conjugates was reported from urine collected from the bladder of RM. These findings suggest that urine is likely the main route by which

RM round goby excretes the putative steroidal pheromone 11-oxo-ETIO conjugate.

3.3 Effect of fluorescein injection

3.3.1 Toxicity

We found no adverse effect of the fluorescein injection on the health and behaviour of male round gobies. Fluorescein was circulated throughout the fish body within a few minutes after injection and the fish skin became yellow/green. The fluorescein started being released through the fish urine and slime within 45 min. Individuals displayed normal feeding and social behaviour. The two largest individuals displayed territorial behaviour immediately after injection. No trace of fluorescein was observed in the urine or skin after 3 days in one male (61.4 g) and after 6 days in another (27.7 g), suggesting that all the dye had been excreted. After 1 month, no dye could be found in the smallest individual's urine (8.6 g), but yellow coloration could still be seen on its skin. No significant change in body weight was observed throughout the test.

3.3.2 Olfactory response

The differential olfactory responses of round goby to fluorescein solutions were compared to the responses to a standard solution of alanine (10^{-5}mol/L) (fig. 1). Responses to the fluorescein solution $C_1=10^{-4}$g/L ranged between 10-38% of the response to Alanine; $C_2=10^{-3}$g/L between 20-39%; $C_4=10^{-1}$g/L between 29-92%. These results suggest that the round goby olfactory system

detects fluorescein when highly concentrated. Therefore, it is unlikely that the presence of fluorescein dye in the experimental tank had a disruptive effect on the results of the present experiment.

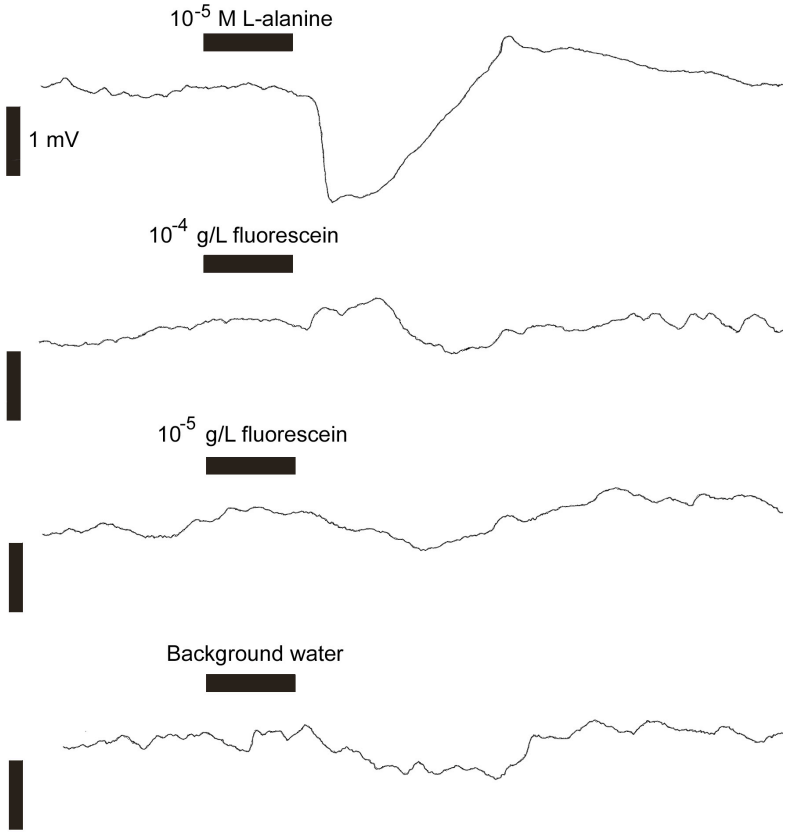

Fig. 1: Electro-olfactogram traces representing the differential olfactory responses of a round goby to solutions of fluorescein (10^{-4} M and 10^{-5} M), a standard solution of L-alanine (10^{-5} M) and background water. For each trace, the horizontal line represents a 5 second delivery of the stated compounds and the vertical bar correspond to a 1 mV calibration.

3.4 Urination behaviour analysis

Overall, males (regardless of their reproductive status) urinated more frequently in the presence of females (regardless of their reproductive status) (mean± SE; N=95) (0.97 ± 0.107) than during isolation periods (0.63 ± 0.094) ($p = 0.0058$) (Fig. 2). The volume of urine released also increased significantly ($p=0.0062$) between isolation (13.00 ± 2.247) and female treatment periods (16.10 ± 1.797) (Fig. 3).

Within male reproductive status (RM and NRM), we observed a similar trend for the general urination activity to increase in the presence of females (Figs.4, .& 5). However, this trend was only partially supported by statistical tests. For instance, in RM, there was no significant difference in urination frequency between female treatments and isolation ($p = 0.114$), but there was a significant rise in the volume of urine released in the presence of a female ($p= 0.022$). On the other hand, NRM did significantly increase their urination frequency in the presence of females ($p = 0.022$) but did not significantly increase their volume of urine released ($p= 0.0914$). Although some of these cases were not statistically significant at the 0.05 level, there was consistent trend for the mean values to increase during female treatment periods.

When a male round goby was introduced into the tank instead of a female, we observed a similar increase in urination by resident males (RM +NRM) compared with the isolation period (Fig. 2). However, this difference was not significant. These additional experiments exhibited higher variability due to a lower number of replicates (N=25 for RM and N=7 for NRM).

Across treatment groups (RF and NRF), female reproductive status had no influence on RM and NRM urination behaviour (Figs. 6 & 7). Although mean urination activity was systematically higher during RF than during NRF treatment, statistical tests showed that NRM and RM did not urinate more frequently or release larger volume of urine in the presence of a RF than in the presence of NRF.

The histograms of the temporal urine release pattern (Fig 8) revealed no particular effect of the treatment order on males' urination behaviour. The operation of introducing or removing a female from the tank did not affect the urine release pattern either.

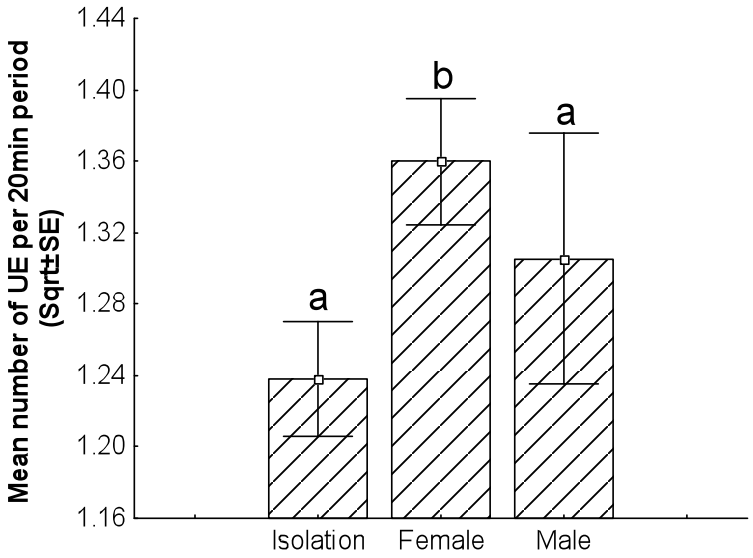

FIG. 2: Mean number of urination events per 20 min period (Sqrt ±SE) by male round gobies (NRM + RM) during isolation period, in the presence of a female conspecific (NRF + RF) or in the presence of a male conspecific. N_{female}=95. N_{male}=64. Letters a and b denote significant differences ($p < 0.05$).

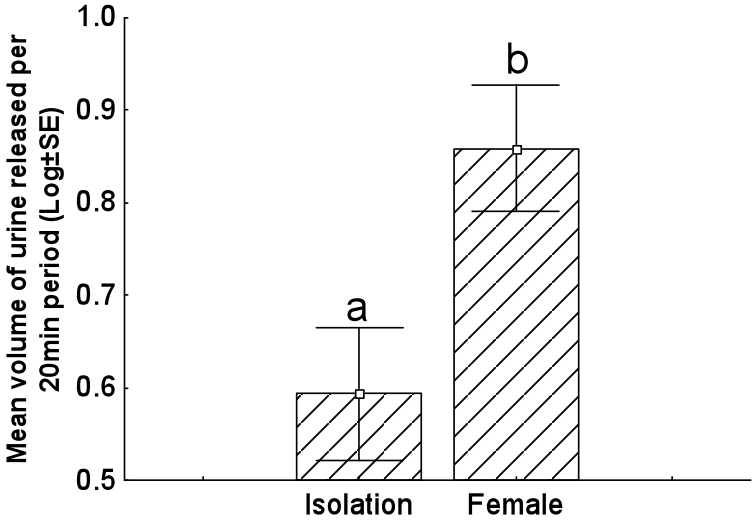

FIG. 3: Mean volume of urine release per 20 min period (Log ± SE) by male round gobies (NRM+RM) during isolation period and in the presence of a female conspecific (NRF+RF). N=95. Letters a and b denote significant differences ($p < 0.05$).

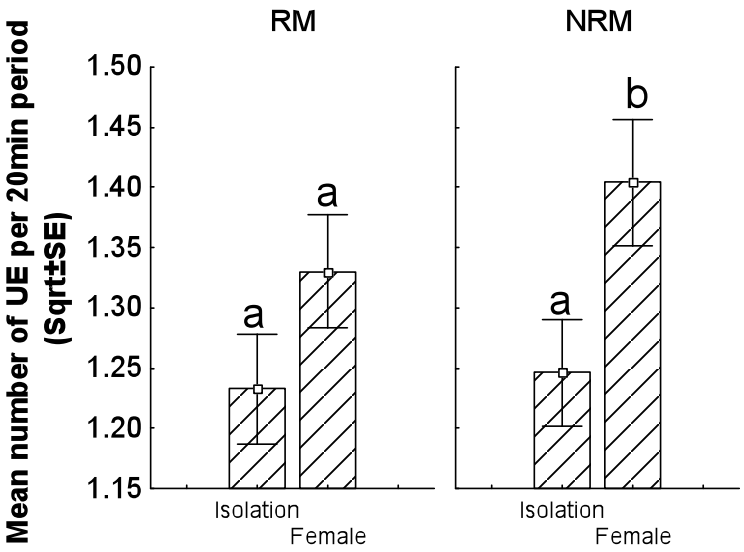

FIG. 4: Mean number of urination events per 20min period (Sqrt ±SE) by NRM and RM during isolation period and in the presence of a female conspecific (NRF + RF). N_{RM}=58. N_{NRM}=39. Letters a and b denote significant differences ($p < 0.05$).

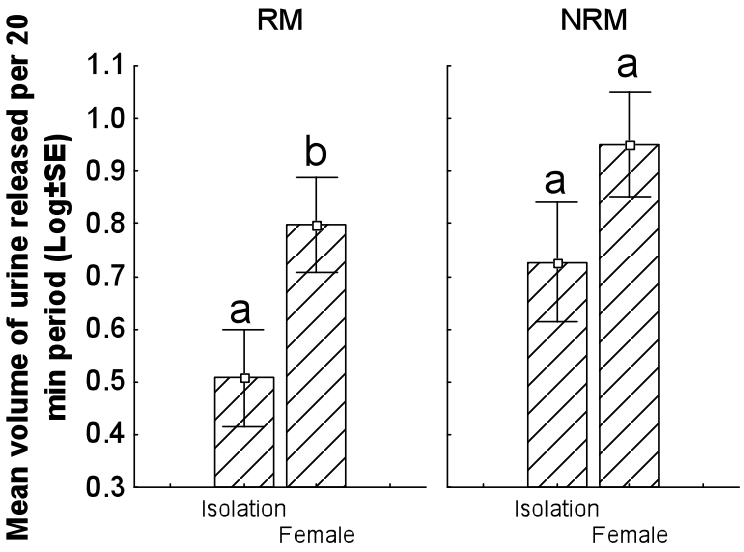

FIG. 5: Mean volume of urine released per 20min period (Log ±SE) by NRM and RM during isolation period and in the presence of a female conspecific (NRF+RF). N_{RM}=58. N_{NRM}=39. Letters a and b denote significant differences ($p < 0.05$).

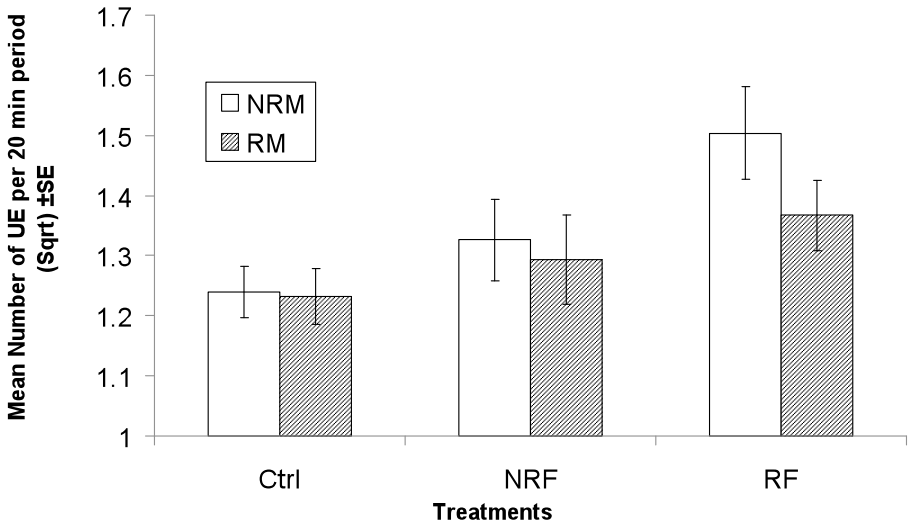

FIG. 6: Mean number of urination events per 20 min period (±SE) by RM and NRM when held in isolation (Ctrl), in the presence of a non-reproductive female conspecific (NRF) and in the presence of a reproductive female conspecific (RF)

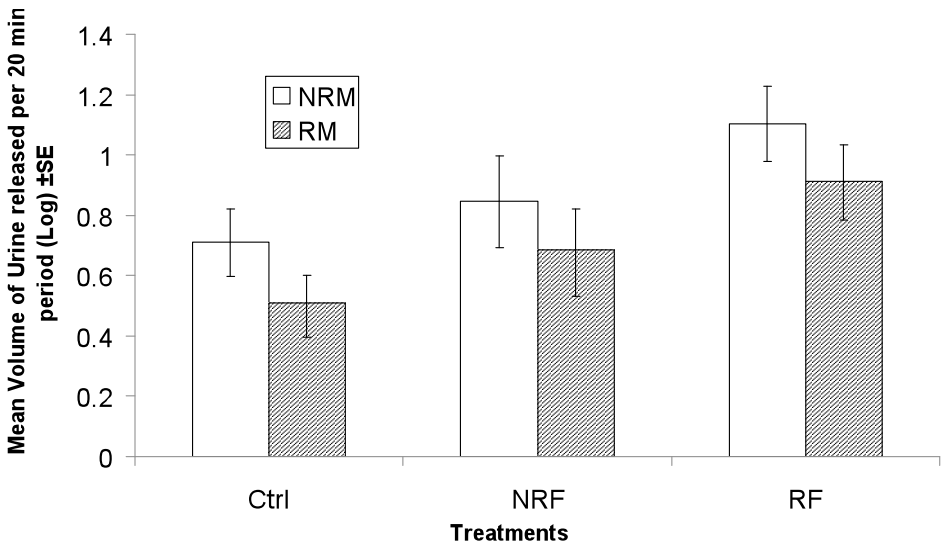

FIG. 7: Mean volume or urine released per 20 min period (log ±SE) by RM and NRM when held in isolation (Ctrl), in the presence of a non-reproductive female (NRF) and in the presence of a reproductive female (RF)

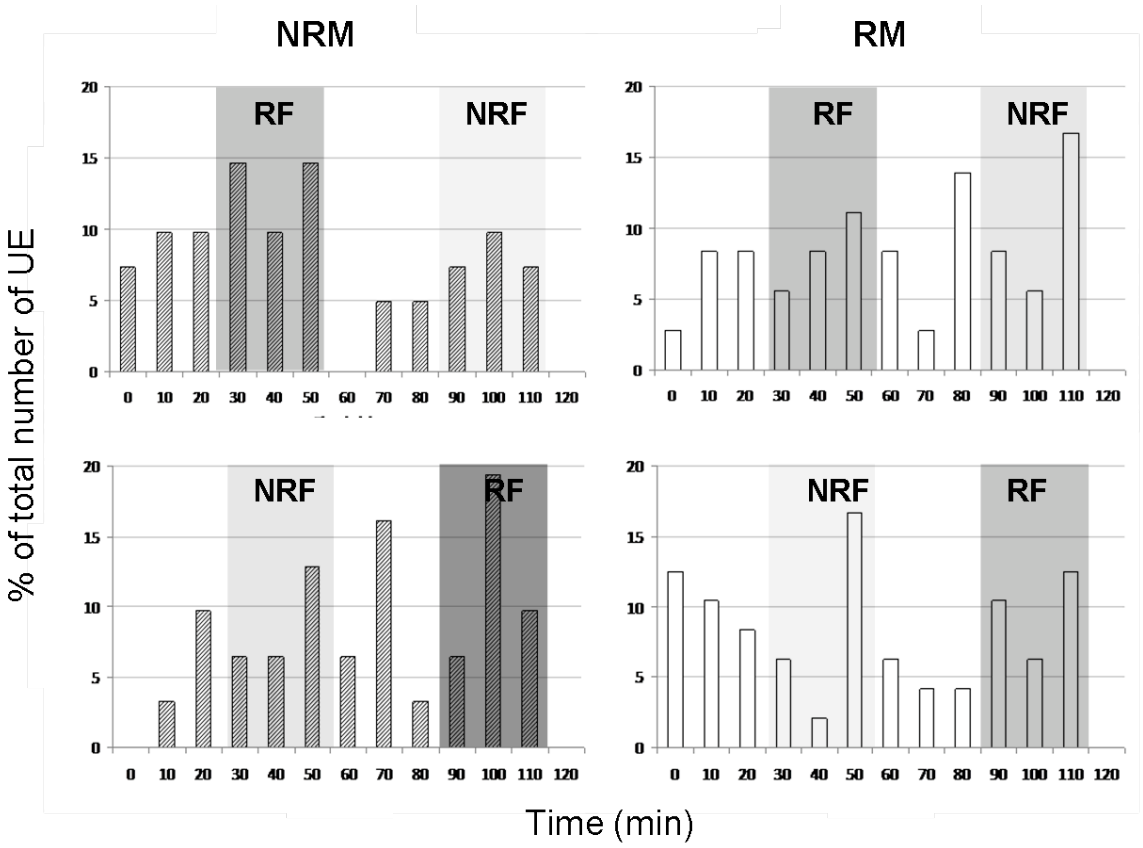

FIG. 8: Temporal urine release pattern by RM and NRM during stimuli (grey area) (NRF, RF) and control periods (white area) (Isolation). T=0min correspond to the beginning of a trial and T=120min correspond to the end of a trail. Column height represents the fraction (in %) of the total number of urination events for each 10 min interval.

4. DISCUSSION

4.1 Evidence that round goby use non-reproductive pheromones.

Our findings showed that round goby males altered their urination activity in the presence of a conspecific. However, contrary to our predictions, variation of the urination activity did not occur in a sexual context. Instead, when a female round goby (regardless of her reproductive status) was introduced into the experimental tank, both reproductive and non-reproductive males urinated more frequently and overall released larger volumes of urine than when isolated. Similarly, round goby males tended to urinate more frequently in the presence of other male conspecifics than in isolation but the difference was not statistically significant. These results contrast with findings from two similar studies which investigated urination activity in male Mozambique tilapia *Oreochromis mossambicus* (Almeida *et al.*, 2005) and in female goldfish *Carassius auratus* (Appelt and Sorensen, 2007). In these studies, fishes increased their urination activity to actively advertize social dominance and/or spawning readiness to potential mates.

Although we showed that urine is likely the main excretion route for putative sex pheromone in the round goby, the fact that both RM and NRM altered their urine release in the presence of conspecifics (regardless of the conspecific sex or reproductive status) suggests that male urine also carries pheromonal products that are unrelated to reproduction. Such species-specific non-reproductive odours have been previously identified in other fishes such as goldfish and common carp (Saglio and Le Martret, 1982; Saglio and Blanc,

1983), Ictalurid catfishes (Bryant and Atema, 1987), Zebrafish *Danio rerio* (Mann et al., 2003), freshwater eel *Anguilla rostrata* (Sorensen, 1986), sea lamprey *Petromyzon marinus* (Li et al., 1995), banded kokopu *Galaxia fasciatus* (Baker and Montgomery, 2001) and also various salmonids (Selset and Doving, 1980; Courteney et al., 1997; Olsen, 1999). In these species, pheromone signals were shown to serve a wide range of biological functions, including anti-predatory and alarm signalling, species and kin recognition in shoaling and dominance hierarchies, species aggregation and migratory attraction (Sorensen and Stacey, 2004).

Because round gobies live in large colonies, it is reasonable to assume that the species would evolve some form of intraspecific communication, enabling individuals to recognize, locate and aggregate with one another. Like shoaling, aggregation of conspecifics is an adaptive cooperation allowing both mature and immature individuals to increase foraging success and reduce predation risk (Pitcher and Parrish, 1993). Others have suggested the possible use of non sex-related pheromones by the round goby (Gammon et al., 2005; Corkum et al. 2006). Behavioural experiments conducted by Gammon et al. (2005) showed that NRF exposed to RF scent spent significantly more time near the odour source compared with control water. Using electro-olfactogram techniques, Belanger et al. (2004) found that both RF and NRF responded to HPLC fractionated odours from RM. On the other hand, Marentette and Corkum (2008) found no clear attraction of male round goby to odours originating from a single male or female conspecific.

Future studies should examine attraction of non-reproductive individuals to odours coming from groups of conspecifics. In a study investigating chemical communication in immature goldfish and common carp, Sisler and Sorensen (2008) found that non-reproductive intraspecific chemical signals in these two closely related species were highly species specific. Goldfish were attracted to conspecific odours, but not to common carp odour, and *vice versa*. Therefore, intraspecific attractants are of special interest because they could be use to control populations of invasive species in North American watersheds such as the common carp and the round goby.

4.2 Elaboration of sex pheromone signals in male round goby.

Results did not support our prediction that only RM should increase their urination in the presence of RF to actively signal spawning readiness. Although mean urination frequency and mean volume of urine released by RM was higher in the presence of RF than in the presence of NRF, the difference was not statistically significant. Similar urination patterns were observed in RM and NRM, thus further rejecting our original hypothesis.

If sex pheromones in RM round gobies had evolved as elaborate communicatory signals, one would have expected RM to significantly increase the potency of their signals when presented with RF by increasing their release of pheromone-laden urine. Although, RF round goby do not develop morphological secondary sexual characters (Miller, 1984), Murphy et al. (2001) and Belanger et al. (2006) found that RM respond to gonadal extracts and

putative pheromones, estrone, from RF. This suggests that RM round gobies, like male Mozambique tilapia (Almeida et al., 2005), can recognize reproductive females based on olfactory cues. Therefore, it is surprising that RM hormonal metabolites would not evolve beyond the level of passive chemical cues, especially since males would benefit from it.

4.3 Possible evolution of sex pheromone signalling in male round goby

We propose that a different evolutionary pathway explains why RM chemical signals are not modulated by changes in the urination activity like in the Mozambique tilapia (Almeida *et al.*, 2005) and the goldfish (Appelt and Sorensen, 2007). Sorensen and Stacey (1999) suggested that the evolution of pheromones is mainly influenced by interactions between conspecifics (*intrinsic* factor) and abiotic pressures (*extrinsic*).

In the round goby, cavity spawning behaviour represents a strong environmental challenge. Although a concealed nest makes it easier for males to protect their eggs against predators or conspecific sneakers, it also makes it more difficult for reproductive females to locate a potential mate. Therefore, male round gobies face a reproductive trade-off between concealing themselves in a nesting cavity and advertising their spawning readiness to the females. We propose that the male concealment in a nesting cavity was a strong extrinsic factor which has driven the evolution of pheromonal signals in the round goby. Therefore, the elaboration of urinary signals has evolved differently in the round goby than in other species in which individuals are not concealed inside

spawning cavities (i.e. Mozambique tilapia, the goldfish). Release of sex pheromone plumes would allow RM round goby to advertise their spawning readiness beyond the entry of their nest without leaving it. Accordingly, a male could increase his reproductive success not only by releasing stronger signals but also by enhancing the dispersion of his reproductive scents to reach a larger number of females. If so, evolution in the round goby must have produced a specialized mechanism allowing RM to enhance their reproductive success. Such a mechanism could exist in the fanning behaviour observed in nesting male round gobies and in many other gobiids and nest-guarding species (reviewed in Meunier et al., 2009). Fanning behaviour was originally thought to serve in cleaning and oxygenating fertilized eggs. However, recent laboratory observations by Meunier et al. (2009) showed that fanning activity by RM round goby starts days before the first egg disposition. This suggests that fanning behaviour might serve to disperse reproductive scents to attract potential mates to the nest. To better understand the level of specialization involved in the sex pheromone signalling by round goby, future studies should focus on the fanning behaviour exhibited by RM and in particular on the possible synchronization between pheromone production, urination activity and fanning activity.

4.4 Summary

This study provides new evidence that round goby may release non-reproductive pheromones during intraspecific encounter. The use of such pheromones have been previously identified in other fishes and has been shown

to play a role in shoaling, species recognition, and conspecific aggregation (Sorensen and Stacey, 2004). The hypothetical use of aggregation pheromones is consistent with the colonial habits of round gobies and the need to maintain a relative proximity among individuals to communicate.

We also found that RM round gobies do not actively advertise their spawning readiness to RF by increasing their release of pheromone-laden urine. These findings contrast with the results of other studies investigating the urination behaviour of other fish species (Almeida *et al.*, 2005; Appelt and Sorensen, 2007). Instead, we suggest that the evolution of sex pheromone signalling in the round goby was driven by extrinsic factors such as the concealment of males within a nesting cavity. Instead of modulating their urination activity, we propose that RM round gobies have evolved other specialized mechanisms such as fanning strokes to better diperse their reproductive scents and advertise their spawning readiness outside the nest without leaving it. Further research is needed to better understand the significance of these mechanisms in the elaboration of pheromonal signals by the round goby and other cavity spawning species.

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CHAPTER 4: QUANTIFICATION OF FANNING CURRENTS BY PARENTAL MALE ROUND GOBY, A CAVITY SPAWNER.

1. INTRODUCTION

Odour tracking behaviours are particularly well developed in aquatic species. In many fishes and crustaceans, individuals use spatio-temporal information contained in odour plumes to navigate toward odour sources such as food or mates (Weissburg, 1997; 2000; Zimmer and Butman, 2000). This mode of communication enables conspecifics to interact and navigate even in dark or turbid environments (Wyatt, 2003; Stacey and Sorensen, 2005). Although olfactory compounds may carry qualitative information about a signaler (i.e., reproductive status in the case of a sex pheromone), the compounds do not provide directional guidance for a receiver. Therefore, olfactory compounds must be released in a structured way so that a receiver can extract the spatio-temporal information needed to locate an odor source (Webster and Weisburg, 2001).

It is unkown how individuals interpret the information contained in olfactory plumes. Different species likely use different strategies (i.e. lobsters and estuarine crabs, see Weissburg, 1997) to track odours. Some receivers exploit variations in the odour concentration to infer a general direction of travel (chemotaxis) (Atema, 1996; Vickers, 2000; Webster and Weissburg, 2001). In other cases, reception of olfactory stimuli triggers a locomotory response of the receiver guided by the direction of the ambient flow (odor-triggered rheotaxis)

(Zimmer et al., 1995; Atema, 1996; Vickers, 2000). Finally, olfactory plume structures may be interpreted using a combination of ambient flow and an odour concentration gradient (odor-gated rheotaxis) (Atema, 1971).

Odour plume structure is strongly affected by the characteristics of the odour released and by the ambient flow surrounding the odour source (Webster and Weissburg, 2001; Weissburg, 2000). Odours can be actively expelled in the environment as pulses or jets (Moore and Atema, 1991; Weissburg and Zimmer-Faust, 1994) or odour can be passively released in a leaky manner (Atema 1966). The active and passive release of odours results in different plume properties (Finelli et al., 1999). Therefore, prior to investigating how individuals track odours in a plume, it is critical to quantify the odour plume release characteristics of the signaler (Webster and Weissburg, 2001).

The round goby *Neogobius melanostomus* is a small benthic fish of the Gobiidae family. During reproduction, male round gobies occupy and defend a nesting cavity –usually a cavity under a rock– to which they attract females for spawning (Miller, 1984; Charlebois et al., 2001). Because round gobies spawn in shallow turbid waters and males are concealed in a nesting cavity, finding a mate is a challenge for members of this species. Yet, field observations showed that a male round goby can guard eggs from up to 15 different females (MacInnis and Corkum, 2000). Male round gobies produce sex pheromones, which attract gravid females to their nest (Arbuckle et al., 2005; Gammon et al., 2005). However, it is unknown how males release and disperse these pheromones and how females exploit the pheromone plume to navigate toward the males.

We investigated the flow field patterns in the vicinity of a round goby nest. Specifically, we described and quantified the fanning currents generated by male round gobies (Wickett and Corkum, 1998; Meunier et al., 2009). Fanning is a common behaviour in fishes with male parental care (present in 30 bony fish families; Blumer, 1979). Fanning consists of the male flipping his fins over the egg mass to oxygenate them and to remove waste (i.e. urine, faeces, sediments) from the nest cavity (Gibbson, 1993). Meunier et al. (2009) showed that fanning activity in the nest-holding male starts before eggs are deposited, suggesting that fanning behaviour might have an additional function of dispersing a sex attractant. We hypothesized that male round gobies use fanning behaviour to actively disperse olfactory compounds in a controlled way that enables gravid females to locate the nest in turbid or dark conditions. We use a flow visualization technique to reveal the fanning current plume structure in a static environment. Because odour plumes are affected by both ambient flow and release characteristics, we specifically selected a static environment to separate the effect of these two factors on the plume structure.

2. METHODS

We used an optical technique adapted from the field of flow dynamic science (Breithaupt and Ayers, 1996; Bergman et al., 2004) to observe and quantify the currents generated by nest guarding male round gobies. This technique, known as Particle Image Velocimetry (PIV) consists of video tracking

the trajectories of small tracer particles previously added into the water and dispersed by the fanning actions of an organism.

2.1. Experimental setup.

Round gobies were collected by angling on the northwest shore of Lake Erie at Leamington Harbour, ON (42°03´N, 82°36´W). After collection, the fish were brought to the laboratory at the University of Windsor. A reproductive male round goby was placed with 4 gravid females in a 35 L tank. The tank was filled with dechlorinated water at 20±1°C (a temperature favorable to round goby reproduction in the laboratory, Meunier et al., 2009) and a 21 cm x 11.5 cm x 4.5 cm PVC shelter to be used as nest by the male. The shelter entrance was rectangular and measured 50 x 45 mm. Five circular holes were drilled at the back of the nest (3 mm) to allow water circulation inside the nest. The water was continuously aerated, insuring a dissolved oxygen level of 75% saturation or more. The bottom of the tank was lined with black aquarium gravel to provide spawning substrate and insure good contrast with the white tracer particles when recording from above the tank. The fish were monitored until spawning occurred. Once eggs were deposited, females were removed from the tank so that the observed currents were generated by the nest-guarding male only.

Polyamide particles were added in the water (Dantec Dynamics, Danemark) to achieve a seeding density in the plane of observation of 3-4 particles/cm^2. These particles (with a relative density of 1.03 g.cm^{-3}), are virtually neutrally buoyant in water. This property allows them to stay in suspension in the

water for an extended period of time. Particle diameter of 50 μm was selected so that the particles had negligible drag in water, yet would still be visible for video recording. The trajectories of the tracer particles were observed in the vertical and the horizontal dimensions. The planes of observation were created by illuminating a thin layer of water (3 mm) using a 500 W slide projector (Kodak Carousel 800) and a narrow slit (0.5 mm) consisting of 2 razor blades mounted in parallel on a photographic slide mount. A color video camera (Hitachi VKC-370) was placed perpendicular to the plane of observation to record the movement of the particles. Footages were recorded on DVD (SONY RDR-GX330). A square ruler was immersed in the field of view of the camera to calculate the scale of the particles' displacement and to correct for any visual distortions due to the perspective.

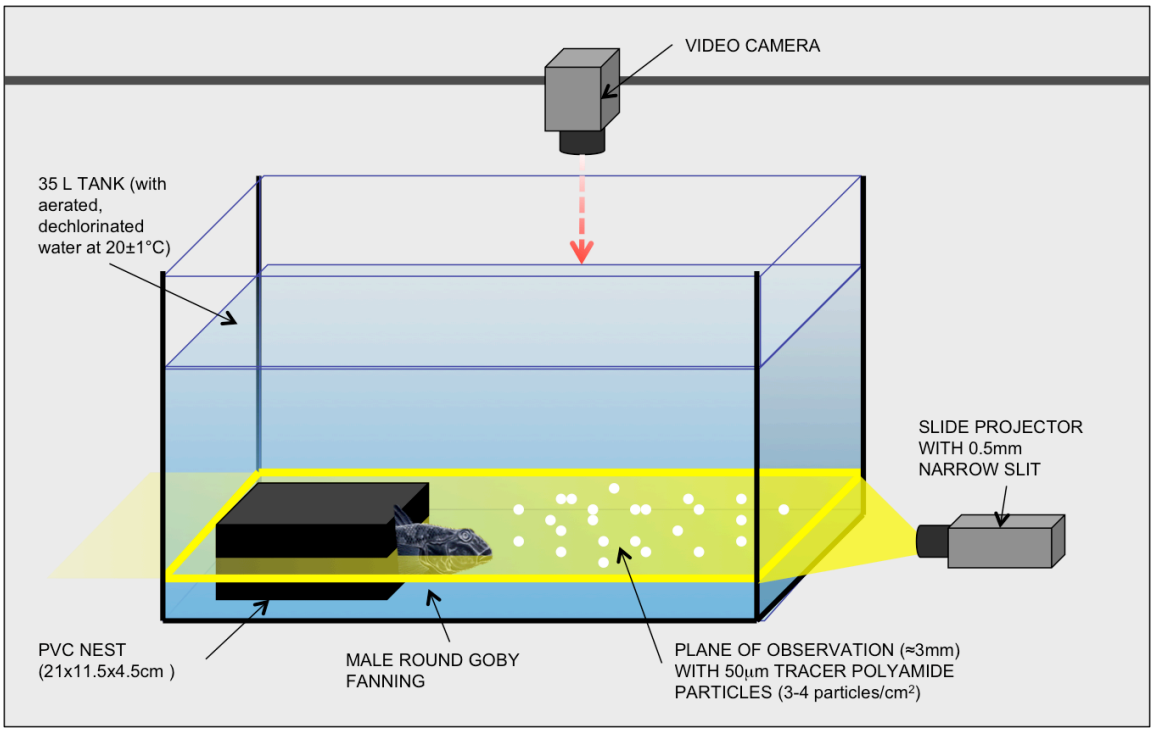

FIG. 1: PIV image analysis setup.

After recording, the videos were transferred to a computer for analysis. The software Image Video Machine (DanDans Digital Media) was used to capture snapshots of a video at regular interval of time (1/25 s). The resulting series of pictures were then processed using JPIV, a Java Particle Image Velocimetry software (Peter Vennemann, http://www.jpiv.vennemann-online.de/). During the PIV image analysis, two consecutive pictures are compared to determine the “start” and “end” position of each tracer particle in the plane of observation. Based on these two positions, the JPIV algorithm calculates the trajectory of the particle and the total displacement during the time laps separating the two pictures.

For the purpose of the analysis, JPIV first divides the plane of observation into rectangular “interrogation windows.” The size of the interrogation window is set by the operator before the analysis and must be chosen according to the seeding density and the relative speed of the particle. The direction and total displacement of particles are then calculated and averaged for each interrogation window and eventually displayed as vectors. The origin of each velocity vector is at the center of the corresponding interrogation window. An example of the resulting vector field is illustrated in Figure 2.

To characterize the currents generated by nest-guarding male round gobies, we monitored the fanning activity of our individual male for one hour, 3 h after egg deposition. We determined the frequency and the duration of the fanning bout and performed a PIV analysis for one randomly selected fanning bout every 10 min of video. In JPIV’s setting frame, we assigned the interrogation

window size at 64 x 64 pixels or 22 x 22 mm (scale 1pixel/0.343mm). For each fanning bout, we extracted 25 pictures per second of video and performed a PIV image analysis between each consecutive picture during the entire duration of the fanning bout.

2.2. Data Analysis

All vector fields were calculated for a given fanning bout (25 vector fields per second) and averaged using the "average vector field" function in JPIV. The resulting average vector field showed the time-averaged velocity of the current surrounding the nest. Variations in the current intensity on the horizontal axis (V_x) were displayed using a color scale. The spatial and temporal data provided by the average vector field were used to measure the various parameters that characterize the currents created by fanning the male round goby. Specifically, we used JPIV's "Velocity profile" tool to estimate the flow of water (cm^2/s) in various regions of the experimental tank.

We estimated the pumping rate of the fanning male round goby by calculating the average flow of water in the vicinity of the nest entrance. Because currents directly at the nest entrance were too turbulent, the velocity profile was calculated at 100 mm from the nest entrance. We estimated the average distance of propagation of the currents generated by the fanning male by measuring the decay of velocity as a function of the distance from the nest. Velocity profiles were measured on the average vector field at 100 mm, 150 mm,

200 mm and 250 mm. We used a quadratic polynomial regression to estimate the decay rate of the velocity as the distance from the nest increased.

$$V_x = a.L^2 + b.L + c$$

where: V_x is the current velocity on the horizontal axis; and,

L is the distance from the nest.

a, b, and c are coefficients of the polynomial equation.

We then extrapolated the propagation distance of particles by solving the regression equation for $V_x = 0$. We estimated the aperture of the current plume by measuring the angle between the right and left edges of the plume. Angle measurements were determined using the image processing software ImageJ (Wayne Rasband, National Institutes of Health, USA). Finally, we calculated the average vorticity (measurement of the local angular rate of rotation in the fluid) in the vicinity of the nest using JPIV's "orthogonal vorticity" tool.

3. RESULTS

3.1. Fanning bouts frequency and Duration

Over a period of one hour, we recorded 50 fanning bouts by the nest-guarding male round goby. The male performed 32 bouts using the caudal fin (CF) and 18 bouts using the pectoral fins (PF). Each fanning bout lasted in average 44 (±5) s for CF and 19 (±4) s for PF. In total, the male spent 49% of the time fanning (39% CF and 10% PF) and the remaining time caring for the eggs (see Meunier et al., 2009).

3.2. Caudal Fanning

Horizontal PIV analysis of six randomly selected CF bouts revealed two general current directions generated during fanning (Fig. 2). Most flow was observed leaving the nest cavity (outflow current), but we also observed one or two inflow streams entering the nest from each side of the nest entrance.

Inflow currents had a time-averaged (±SE) maximal velocity of 5.4 (±0.78) $mm.s^{-1}$. The location of inflow currents varied between fanning bouts. When the male performed fanning strokes on the right side of the nest entrance, inbound currents entered the nest on the left side and *vice versa* (e.g. Fig. 2B). When the male performed fanning strokes at the centre of the nest entrance, we observed inbound currents on each side of the nest entrance (e.g. Fig. 2C).

Steady strokes of the caudal fin generated a strong outflow current originating at the nest entrance and propagating outside the field of view of our camera. Outflow currents were characterized by a uniform direction of the velocity vectors. The time averaged maximal velocity at the centre of the flow of 13.84 (±0.78) $mm.s^{-1}$ and sharp velocity gradients at the edges. Overall, direction and shape of the outflow current plumes varied between fanning bouts. The average opening angle of these plumes on the horizontal plane was 57.6° (±1.8). On the vertical plane, PIV analysis revealed that the outbound currents had a limited vertical propagation (Fig 3). Most of the flow occurred in a 50 mm water layer at the bottom of the experimental tank.

Analysis of the flow vorticity revealed two distinct areas of turbulence in the nest vicinity (Fig 4). Turbulence was created by velocity shears at the interface between the outflow current and the stagnant ambient water. In the turbulent area at the left edge of the outflow current plume, the mean vorticity was 0.066 (±0.002) (anti-clockwise rotation). At the right edge of the outbound current, mean vorticity was -0.063 (±0.002) (clockwise rotation). The mean vorticity at the center of the outflow current was null. This pattern was consistent among fanning bouts.

3.3. Pectoral fanning

In contrast with CF bouts, horizontal PIV analysis of the currents generated by PF bouts revealed no flow exiting the nest cavity (Fig 5). Pectoral fin strokes did not create a well-defined water circulation as seen with CF. Instead we observed a weak stream entering the nest with a maximal velocity of 4.11 $mm.s^{-1}$.

3.4. Estimated caudal fanning pumping rate

Velocity profiles at 100 mm from the nest entrance revealed an average flow velocity in the observation plane of 7.7 (±0.66) $mm.s^{-1}$. Assuming that the current plume is uniform in the z dimension and is constrained by the nest opening dimensions (48 cm^2), we estimated the average CF pumping rate of the male round goby at 36.7±3.2 $cm^3. s^{-1}$.

3.5. Estimated distance of propagation

Velocity profiles calculated at increasing distances from the nest revealed that a maximum flow rate in the current plume was reached at 150 mm from the nest. Beyond 150 mm, flow rate in the current plume started to decrease. This pattern was consistant between fanning bouts (Fig 6). A polynomial regression showed that the velocity decay in the current plume along the x axis followed the equation:

$$V=-2.5 \times 10^{-4} L^2 + 7.84 \times 10^{-2} L + 2.33 \qquad \text{(Fig. 6)}$$

Where V is the velocity and L the distance from the nest. The regression equation accounted for 18.8% of the variance of velocity between fanning bouts and the coefficient of correlation R was 0.43. Based on this regression equation, outbound currents generated by the fanning male round goby propagated to a distance of 344 mm from the nest entrance.

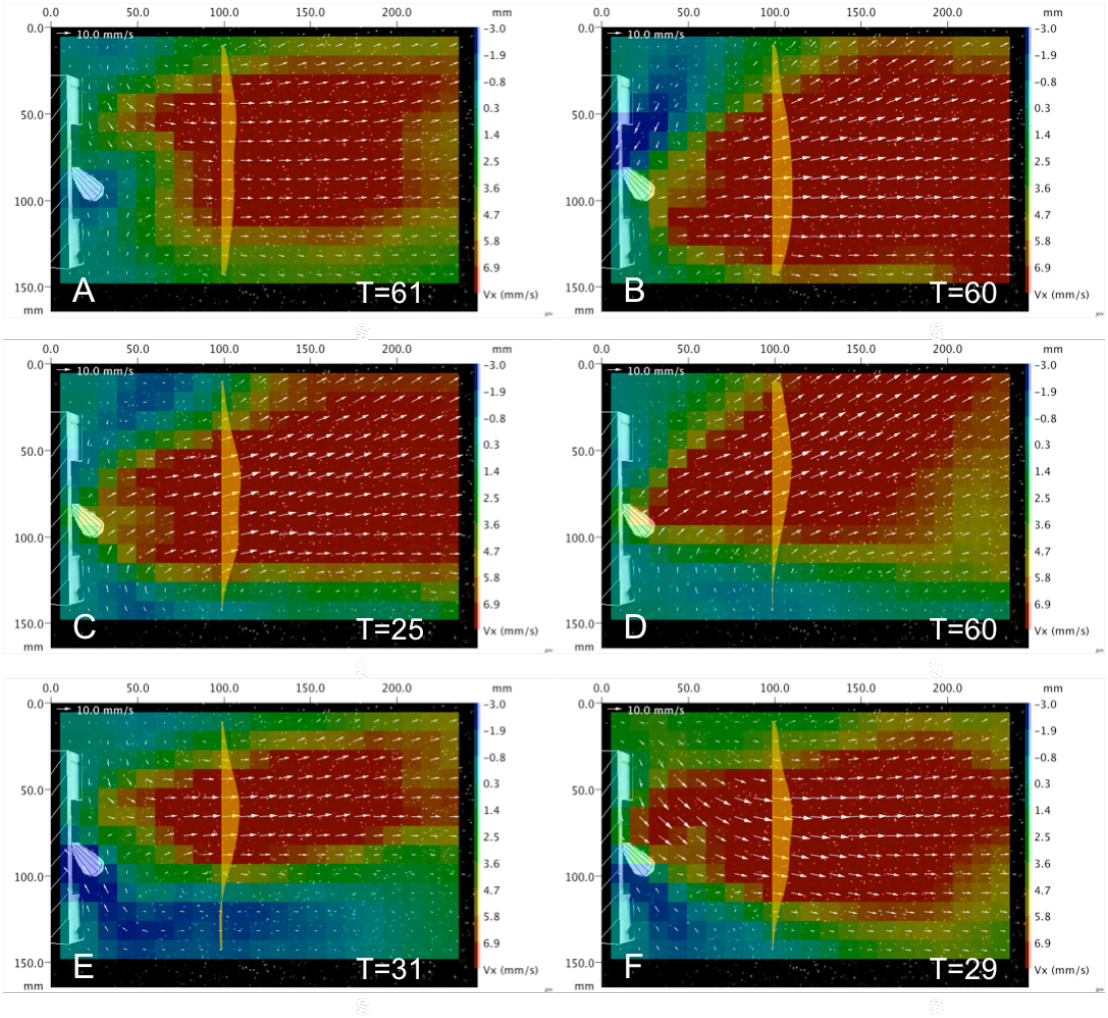

FIG. 2: Horizontal flow visualization: Time-averaged velocity fields for 6 different caudal fanning bouts. The nest entrance is on the left side of each diagram. The scale of the coordinate system is 1 pixel/0.343 mm (Top and left axis). The color code represents the velocity of the particles in the X direction. The temporal resolution is 0.04 s (25 frames per sec). T is the duration of the fanning bout in seconds. The orange shape is the velocity profile at 100mm from the nest entrance. The surface area of the velocity profile is proportional to the in-plane flow per unit length in the Z-direction.

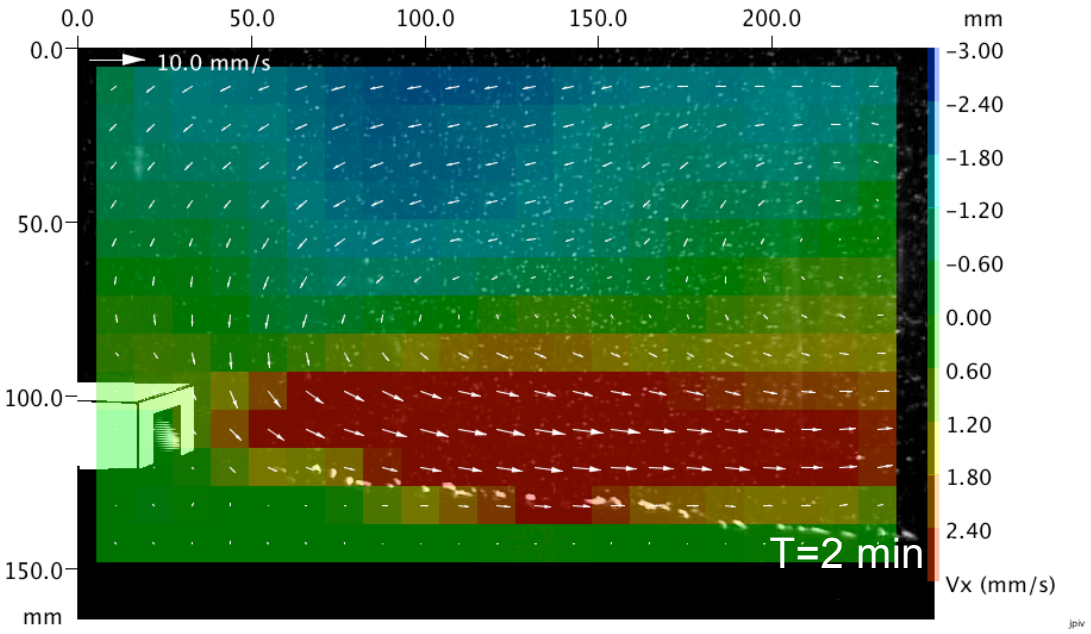

FIG. 3: Vertical flow visualization: Time-averaged velocity field during a caudal fanning bout. The nest entrance is on the left side of each diagram. The scale of the coordinate system is 1 pixel/0.343 mm (top and left axis). The temporal resolution is 0.04 s (25 frames per sec). T is the duration of the fanning bout. The color code represents the time-averaged velocity of the particles in the X dimension.

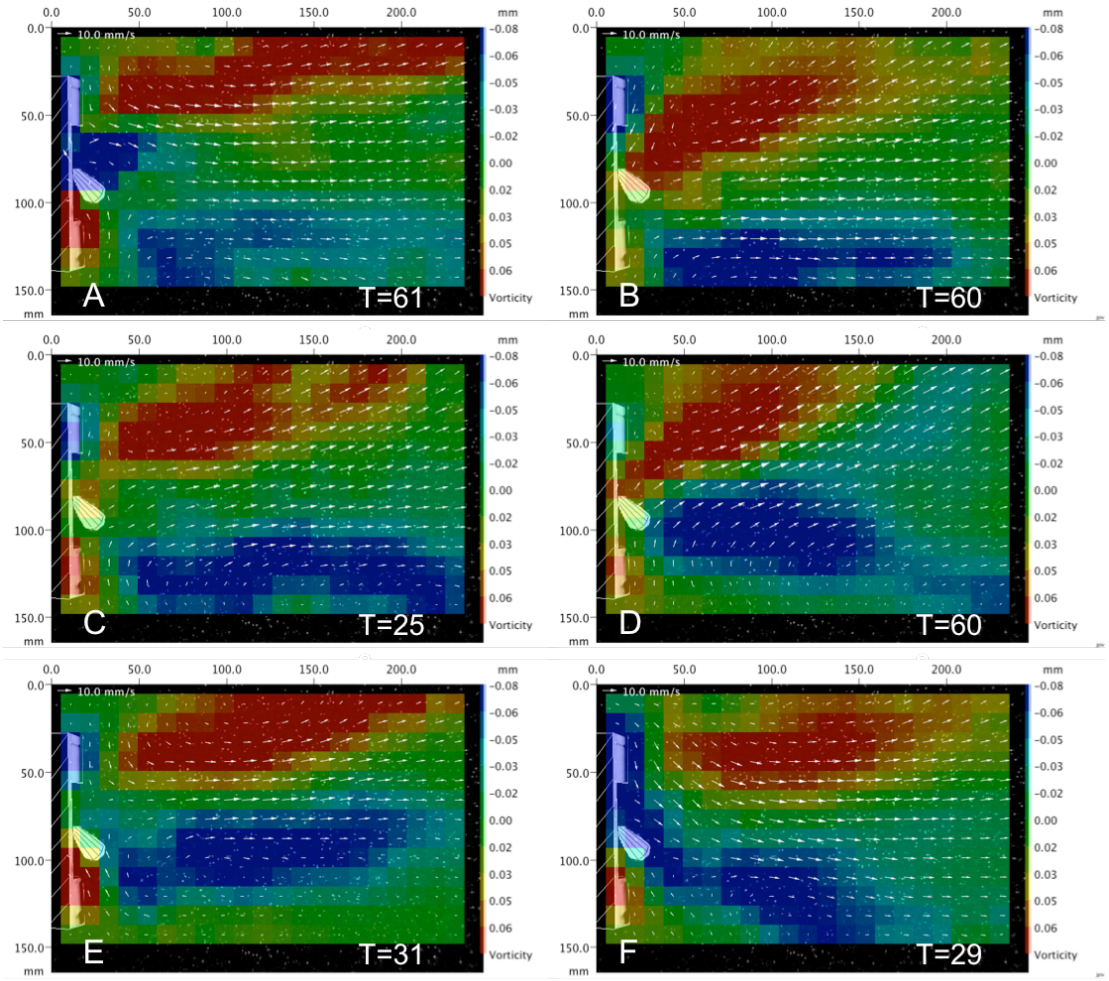

FIG. 4: Horizontal flow visualization: Time-averaged vorticity fields for 6 different caudal fanning bouts. The nest entry is on the left side of each diagram. The scale of the coordinate system is 1 pixel/0.343 mm (top and left axis). The temporal resolution is 0.04 s (25 frames per sec). T is the duration of the fanning bout in seconds. The color code represents the orthogonal vorticity of the flow.

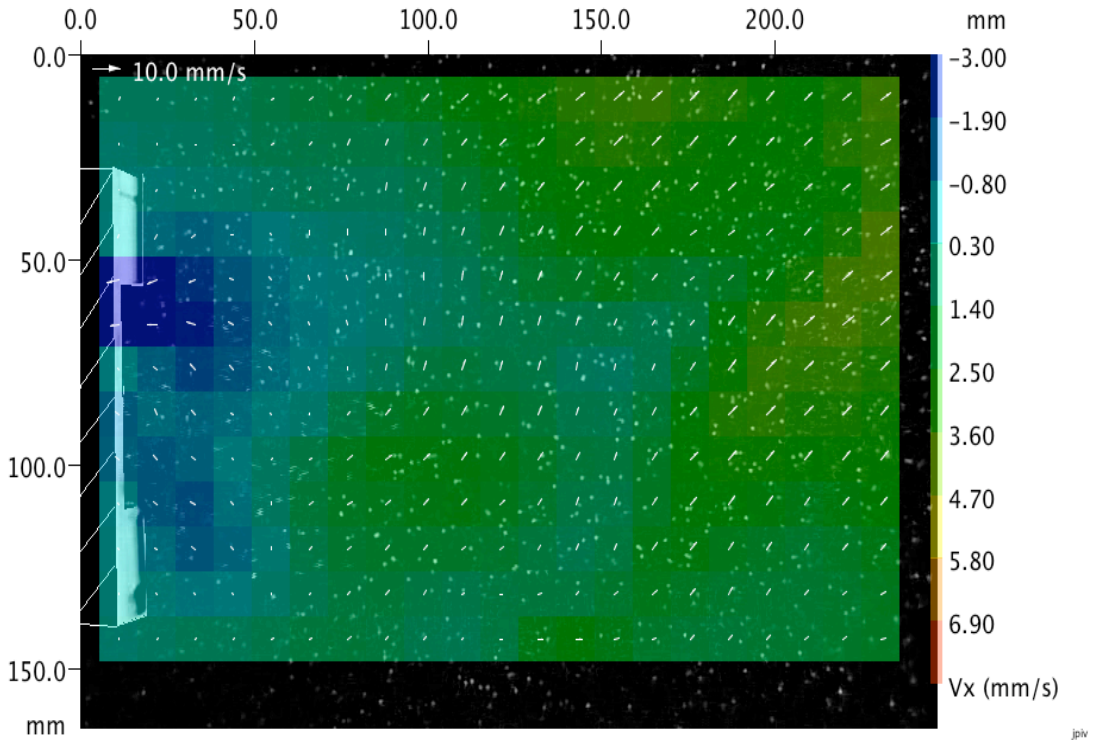

FIG. 5: Horizontal flow visualization: Time-averaged velocity fields for a pectoral fanning bout. The nest entry is on the left side of the diagram. The scale of the coordinate system is 1pixel/0.343mm (top and left axis). The temporal resolution is 0.04 s (25 frames per sec). The color code represents the velocity of the particles in the X dimension. T is the duration of the fanning bout.

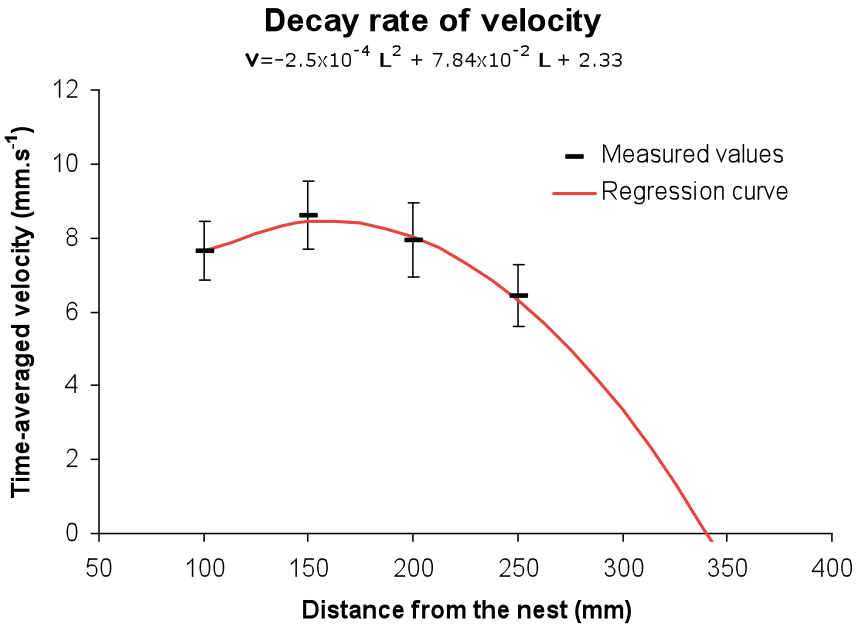

FIG. 6: Decay rate of time-averaged velocity (V) function of the distance from the nest (L). A polynomial regression showed that the time-averaged velocity of the fanning currents follows the equation: $V=-2.5 \times 10^{-4} L^2 + 7.84 \times 10^{-2} L + 2.33$. The estimated distance of propagation of fanning currents is 344 mm.

4. DISCUSSION

Our results showed that fanning strokes performed by a nest–guarding male round goby created strong circulatory currents in the nest vicinity. We demonstrated that caudal fanning generates currents entering and exiting the nest. Pectoral fanning also generated an inflow current but no outflow current. These results are consistent with the egg oxygenation and waste removal function proposed by others for the fanning behaviour of many cavity-spawning fishes (Jones and Reynolds, 1999; Gibson 1993).

The round goby male pumped “waste water” out of the nest by repeated tail flippings. Low pressure resulting from water leaving the nest cavity generates currents of opposite direction, bringing “fresh water” inside the nest. With some assumptions, we estimated that the male used in this study pumped an average 36.7 $mL.s^{-1}$ during each fanning bout. However, this pumping rate likely varies with male characteristics (larger males likely pump at a higher rate), age of eggs (i.e. time after egg deposition) and environmental factors. In the sand goby *Pomatoschistus minutus* (Lindström and Wennström 1994, Järvi-Laturi *et al.* 2008), freshwater goby *Padogobius martensii* (Toricelli *et al.,* 1984), two-spotted goby *Gobiusculus flavescens* (Skolbekken and Utne-Palm, 2001) and a landlocked goby *Rhinogobius sp* (the orange form) (Maruyama et al., 2008), fanning activity is positively correlated with egg age and/or negatively correlated with the oxygen concentration.

Interestingly, we found that pectoral fanning does not produce a strong

water circulation in comparison with caudal fanning. Instead, we observed a weak current entering the nest and did not detect any current exiting the nest. This result suggests that the waving of the pectoral fin in a typical figure 8 pattern (Meunier et al., 2009) does not serve the same function as the flipping of the caudal fin in the round goby. The hydrodynamic effect of pectoral fins waves is not expressed outside the nest and thus must be expressed inside the nest cavity. Because we observed that caudal fanning always followed pectoral fanning, we suggest that male round gobies use pectoral fanning to stir solid wastes that settled in the nest cavity (i.e. faeces, sediments) and facilitate their transport by the exiting current generated during caudal fanning. Similarly, male bluegills *Lepomis macrochirus* fan theirs eggs with their pectoral fins, not to aerate them (sunfish eggs hatch well in low oxygen conditions), but likely to reduce siltation (Breder, 1936).

Although the present study was conducted only 3 h after egg deposition and in relatively high oxygen conditions (75% saturation), the focal male spent as much as 49% of his time fanning. This value is much higher than those reported in other gobiids with freshly laid eggs in high oxygen concentration (*Pomatoschistus minutus*, 16% (Lindstrom and Wennstrom, 1994; Jones and Reynolds, 1998); *Gobiusculus flavescens* , 18% (Skolbekken and Utne-Palm, 2001); *Rhinogobius sp*: 32% (Maruyama et al., 2008)). Meunier et al. (2009) also reported a high fanning activity by spawning male round gobies in similar conditions. These studies suggest that male fanning behaviour may play a secondary role in the biology of the round goby such as the dispersal of olfactory

signals.

Our results showed that currents produced by a nest-guarding male round goby can maximize the dispersion of chemical signals. The male was able to propagate a current as far as 35 cm from his nest, over a surface area of 987 cm^2 and had a limited vertical (50mm) propagation. Although, these values likely vary with male's characteristics and vigour at fanning, it is reasonable to assume that male round gobies exploit these currents to better "cast" their reproductive scents and attract a larger number of mates. Laboratory observations by Meunier et al. (2009) showed that fanning activity of breeding male round gobies started well before the first egg deposition. This reinforces the case that male fanning behaviour plays a key role in the mate attraction process in the round goby. The association of fanning and mate attraction has been previously documented in the sand goby, whose males increase their fanning efforts in the presence of potential mates (Pampoulie et al. 2003) and in the three-spined stickleback *Gasterosteus aculeatus* where males perform courtship fanning even in the absence of eggs in their nest (Sevenster, 1961).

Although the association of nest fanning and sex pheromone dispersal has yet to be reported in fish, some aquatic species are known to generate their own excretory currents to disperse odours (Atema, 1996). For instance, male and female lobsters can inject urine into their gill current to disperse pheromonal signals during courtship (Atema, 1986; Atema, 1995). Male lobsters also use pleopod fanning at the shelter entrance to disperse odours into the environment and advertise their reproductive status (Atema, 1986). The release velocity of an

odour and the distance of the odour source over the substrate dramatically affect the character of a dispersion plume (Webster and Weissburg, 2001). In the case of an active release (release velocity superior to ambient flow velocity) with a source sitting on the substrate (i.e. a male round goby releasing urine and fanning), experiment showed that high velocity shear results in more homogenous odour concentration in the dispersion plume (Webster and Weissburg, 2001). Thus, fanning behaviour could serve to homogenize odor plumes and enhance their detectability by receivers.

Beyond the dispersal of olfactory signals, fanning currents themselves carry directional information that can be exploited by receivers to navigate toward an odour source (Atema, 1996). Our results showed that time-averaged current plumes generated by male round gobies have a constant pattern that is characterized by a strong linear flow at the center of the plume and areas of turbulence at the edges. In these conditions, it is likely that round goby females use a combination of olfactory and mechanical cues –referred as chemo-rheotaxis (Atema, 1996; Webster and Weissburg, 2001)– to find mates in turbid or dark environments. In the slow moving waters inhabited by the round goby (Miller, 1984; Skazkina, 1972; Charlebois et al., 2001), an active dispersal area dominated by a sharp flavored current likely provides better directional information than loose patches of odours passively drifting in the ambient flow (Atema, 1996). Round gobies are particularly well suited for the detection of modulations in the ambient flow pattern. The abundance of superficial neuromasts throughout the body of round gobies makes them more sensitive to

hydro-mechanical stimuli than other fishes with neuromasts enclosed in the lateral line canal (Jones and Janssen, 1992; Hoekstra and Janssen, 1985, 1986; Janssen et al., 1990). Finally, the release velocity of an odour and the distance of the odour source over the substrate dramatically affect the character of the dispersion plume (Webster and Weissburg, 2001). In the case of an active release (release velocity superior to ambient flow velocity) with a source sitting on the substrate, experiment showed that high velocity shear results in more homogenous odour concentration in the dispersion plume (Webster and Weissburg, 2001).

5. CONCLUSION AND FUTURE DIRECTIONS

In this study we showed that a simple and low cost technique adapted from the field of fluid mechanics could be applied effectively to study the biological flow fields in the vicinity of round goby nests.

We demonstrated that fanning behaviour by a nesting male round goby produces the effect proposed by other studies. Specifically a water circulation which conveys well oxygenated water inside the nest and flushes wastewater outside the spawning cavity. We also provide the first evidence to support the hypothesis that fanning behaviour plays an important role in courtship behaviour of male round gobies. In addition to the active dispersal of olfactory compounds, fanning currents could also serve as hydro-mechanical stimuli to carry directional information enabling a female to locate a nesting male. Hence, we propose that

female round gobies use a combination of chemotaxis and rheotaxis to find potential mates or to move within range of other male sensory signals (i.e. visual, vocal).

Further research is needed to fully understand the role of fanning behaviour in the mate attraction process in the round goby. Variations in the fanning activity between different males (i.e. age, morphometry) before and after spawning need to be investigated. Questions, such as how males orchestrate fanning behaviour and pheromone release (i.e. urine release) and how females use these signals to navigate needs to be answered. Finally, to fully understand the structure of odour plumes released by male round gobies, future investigations should define and quantify the typical ambient flow dynamic in the round goby habitat.

Most of these questions can be addressed using the same PIV technique described in this experiment. Yet, some simple modifications could increase the precision and accuracy of future results. A larger experimental flume would avoid the creation of recirculation currents by the reflection of the fanning current against the end of the flume. A high-speed camera would enable the operator to sample more than 25 frames per seconds so that faster fanning currents could be analysed at a finer scale. Finally, a wide-angle lens would enable the visualization of the entire fanning plume and better assess the range of attraction of sex pheromones released by the male round goby.

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CHAPTER 5: GENERAL DISCUSSION

In studying chemical communication, it is important to characterize how olfactory signals are released and dispersed by the producer before investigating how these signals are interpreted by the receiver (Atema, 1996; Vickers, 2000; Webster and Weissburg, 2001). The study of a sensory system function and operation first requires one to characterize the nature of the input to the system (Webster and Weissburg, 2001). In the case of chemically mediated guidance, a receiver exploits spatio-temporal information contained in odor plumes to locate and navigate toward an odour source. The patterns and distribution of odours within plumes are mainly affected by the ambient flow and by the release characteristics of the originator of the signal (Webster and Weissburg, 2001). Thus, it is important to characterize ambient flow and release characteristics of the signaler to understand how receivers exploit odor plumes to locate and navigate toward distant objects.

In the present study, we characterized the release and dispersion of olfactory urine signals by nesting male round gobies. We determined that urine is the main excretion route for sex pheromones in male round gobies. We found that male round gobies release pheromone-laden urine spontaneously and do not modulate their urination activity in the presence of reproductive females. In other words, urine releases are not triggered by reproductive female stimuli. This result is consistent with the idea that nesting male round gobies release pheromones as chemical guidance cues to attract remote females to the nest in

turbid water. In such conditions and because reproductive male round gobies are concealed in their nest, the males likely see, hear or smell gravid females within a short range. Thus, males initiate communication by releasing urinary sex pheromones and attract gravid females within range of other sensory cues. The fact that reproductive males do not modulate their urine release in the presence of a reproductive female suggests that male urinary signals are not active signals intended to court a nearby individual. Instead, urinary signals are passively released to create an odor track enabling distant reproductive females to navigate toward the nesting cavity.

Based on our research findings, we suggest that a reproductive round goby male may enhance his reproductive success by increasing the potency of his olfactory signal and/or by enhancing the dispersion of his pheromones. In other words, olfactory signal potency and dispersion must vary substantially according to male reproductive qualities. The study of male round goby urination activity (chapter 3) confirmed that there were significant variations in the volume and frequency of urine releases between individuals of the same reproductive status. During a typical behavioural assay, males urinated up to 25 times in 20 min. Male urine samples collected over 4 h contained up to 117 ηg of putative sex pheromone 11-oxo-ETIO-conjugate. By increasing the frequency or volume of urine release, a male could increase the potency of his signal and thus increase the chances that a nearby female would detect his presence. Alternatively, by enhancing the dispersion of his pheromones, a higher quality male could increase his “area of influence” and reach more potential mates

nearby. Because round gobies inhabit slow moving waters (Millers, 1984) of the boundary layer (the water layer closest to the substrate) (Denny, 1993; Vogel, 1993), ambient flow is likely a poor vector for the horizontal transport of pheromonal attractants (Weissburg, 1997, 2000; Vickers, 2000). Thus, we hypothesized that male round gobies must have evolved a mechanism enabling reproductive individuals to actively "cast" their smell at a greater distance from the nest.

To identify such a dispersal mechanism, we investigated the reproductive behaviour of round goby. We presented the first detailed description of the round goby spawning behaviour and defined how nesting males allocated their time and energy to different reproductive activities (Chapter 2). Specifically, we found that parental activities by male round gobies are dominated by fanning behaviour. In cavity-spawning species (this breeding system is characterized by many Gobiidae, Cottidae and others), fanning is believed to enhance egg survival by bringing oxygen and removing wastes from the nesting cavities (Gibson, 1993; Torricelli *et al.*, 1984; Jones and Reynolds 1999a; Takegaki and Nakazono 1999; Maruyama *et al.* 2008). Interestingly, we observed that round goby fanning behaviour starts well before the first egg is deposited (chapter 2) and is more intense than in other gobiids with freshly laid eggs and in high oxygen conditions (discussed in chapter 4) (Lindstrom and Wennstrom, 1994; Jones and Reynolds, 1998; Skolbekken and Utne-Palm, 2001; Maruyama et al., 2008). These novel observations suggest that fanning in the round goby must

have an additional function, other than nest aeration, and is likely involved in the mate attraction process.

We proposed that fanning currents are generated by nesting male round gobies to enhance the dispersal of pheromonal products and to attract mates to their nest. Thus, fanning could be an adaptive behaviour whereby nesting males combine parental care (specifically nest maintenance) and mate attraction. From an evolutionary point of view, this raises the question of which function evolved first. Fanning is a common behaviour shared by at least 30 bony fish families with parental care (Blumer, 1979). The involvement of fanning in the mate attraction process has been shown in at least two species: 1. Male sand gobies increase their fanning efforts in the presence of potential mates (Pampoulie et al. 2003). 2. Male three-spined stickleback *Gasterosteus aculeatus* perform courtship fanning even in the absence of eggs in their nest (Sevenster, 1961). Some crustaceans also produce their own current to disperse odours. For instance, male lobsters *Homarus americanus* use pleopod fanning at the shelter entrance to disperse odours into the environment and advertise their reproductive status (Atema, 1986). However, further research is needed to investigate the association of fanning and pheromone dispersion in fish.

Odours can be actively expelled in the environment as pulses or jets (Moore and Atema, 1991; Weissburg and Zimmer-Faust, 1994) or odours can be passively released in a leaky manner (Atema 1966). The release velocity of an odour and the distance of the odour source over the substrate dramatically affect the character of a dispersion plume (Webster and Weissburg, 2001). In the case

of an active release (release velocity superior to ambient flow velocity) with a source sitting on the substrate (i.e. a male round goby releasing urine and fanning), experiments showed that high velocity shear results in more homogenous odour concentration in the dispersion plume (Webster and Weissburg, 2001). Thus, fanning behaviour could serve to homogenize odor plumes and enhance their detection by receivers.

We used a flow visualization technique (particle image velocimetry) to investigate the fanning currents generated by a nesting male round goby. We found that the characteristics of these currents were consistent with the function proposed above. The waving action of the male's tail generated strong currents that propagated from the nest. Currents produced by an experimental male propagated horizontally as far as 35 cm from the nest entrance and had limited vertical propagation. We estimated that the male could expel up to 36.7 $mL.s^{-1}$ of water from his nest during a fanning bout. The limited vertical propagation of these currents results in most of the fanning energy to be dissipated in the direction of the longitudinal axis. This supports the idea that male round gobies use fanning currents to "cast their odour" as far as possible from their nest and as close as possible to the substrate (round gobies are benthic) to reach remote reproductive females. We found that the time-averaged current plumes had a constant pattern characterized by a strong linear flow at the center of the plume and areas of turbulence at the edges. We concluded that, in addition to enhancing the dispersal of pheromones, these currents could also be exploited by females to infer a direction of travel and navigate toward males (Atema, 1996;

Webster and Weissburg, 2001). In fact, flow can provide an additional source of information that can be integrated with the detection of olfactory signals (Vickers, 2000). In the slow moving waters inhabited by round goby (Miller, 1984; Skazkina, 1972; Charlebois et al., 2001), an active dispersal area dominated by a sharp flavored current likely provides better directional information than loose patches of odours passively drifting in the ambient flow (Atema, 1996). The combination of chemotactic (odor mediated) and rheotactic (flow mediated) mechanisms to locate odour source appears to be common in many fish species (Vickers, 2000). For instance, rainbow trout *Oncorhynchus mykiss* (Emanuel and Dodson, 1979) and sharks (Hodgson and Mathewson, 1971) use odour detection and up-current movement to locate sources of odour. However, for a substrate-dwelling species such as round goby, benthic crustaceans are perhaps better comparative models. Specifically, chemotaxis and rheotaxis in crabs and lobsters have been particularly well studied (Weissburg and Zimmer-Faust, 1993, 1994; Zimmer-Faust et al., 1995; Moore et al., 1991). Experimental designs and results of these studies could inspire future studies to explore odour and flow-modulated navigation in female round gobies.

Overall, this study provided the first description of the release and the dispersion of urinary signals by nesting male round gobies. The significance of these results resides in the understanding and the characterization of the round goby pheromone communication system. These results could find a practical application in the design of pheromone baited traps to control the spread of this

invasive species into new areas. We developed new techniques for rearing round gobies in the laboratory, visualizing urinary signals and quantifying biological flow fields. These methods will find direct applications in future studies investigating pheromone signaling in the round goby and other aquatic species.

Additionally, our research findings raise new questions about the evolution of sex pheromone signals in fish. In contrast with female goldfish (Appelt and Sorensen, 2007) and male Mozambique tilapia (Miranda et al., 2005), reproductive male round gobies do not modulate their urinary signals in the presence of a potential mate. An explanation could be that urinary signals do not perform the same function in these species. In the study of pheromonal systems, it is important to make the distinction between sex attractants (signals that lure conspecifics in the general vicinity of the male) and courtship pheromones that function once the mate has been identified and preliminary courtship behaviour are initiated (Houck, 1987; Houck et al., 2007). Male Mozambique tilapia and female goldfish release a urine signal to advertise their quality as a mate, social dominance and spawning readiness (Miranda et al., 2005; Appelt and Sorensen, 2007). Thus, the function of urinary signals in Mozambique tilapia and goldfish is related to courtship and mate assessment. On the other hand, male round gobies release urinary signals to attract female individuals that otherwise could not detect the males because of the nest concealment and the ambient turbidity. Thus, the function of urinary signals in male round gobies appears to be mate attraction. We proposed that the evolution of pheromone signals in cavity spawners was mainly driven by extrinsic factors (i.e. the male concealment in a

nesting cavity) instead of intrinsic factors (i.e. intra-specific interaction). For cavity spawning species with parental care, finding a mate can be challenging. Nest guarding males face a reproductive trade-off between providing care to the eggs inside the nest and advertising their spawning readiness outside the nest to attract females to spawn. Thus, some cavity spawning species (if not all) must have evolved mechanisms enabling males to advertise their presence without leaving the nest. This study showed that in the round goby, pheromonal attractants are released through the urine and actively dispersed by reproductive males from within the nesting cavity.

Further research is needed to fully characterize the round goby pheromonal system. The chemical identification of the active compounds composing male round goby pheromonal blend is in progress (B. Zielinski, University of Windsor). A colleague, Dr. J. Ackerman (University of Guelph), is investigating the influence of ambient flow on the dispersion of pheromone signals in typical round goby riverine habitats. The results of Dr. J. Ackerman's study will help to understand how round goby pheromones are dispersed after dissipation of the male fanning currents. Ultimately, the characterization of both fanning currents and ambient flow in round goby habitat will help to determine the range of attraction of male round goby pheromone.

Future studies should examine how urine release and fanning behaviour are synchronized, how females exploit pheromone plumes to navigate toward a nest and how other signals (i.e., vocal, visual) are involved in the mate attraction and mate selection processes in the round goby. The synchronization of urine

signals and fanning currents could be investigated using a combination of the urine release visualization and flow field visualization techniques developed in this study (chapter 3 and 4). The transport of urine signals by fanning currents could be documented by using a fluorescein injected male in a tank equipped for PIV analysis and UV light. New techniques such as the planar laser-induced fluorescence (LIF) technique (see Weissburg, 2000) could also be used to quantify concentration of olfactory compounds within the dispersion plume. Characterization of the concentration patterns within the dispersion plume would enable future studies to understand how female round gobies exploit patterns in male odour plumes to navigate toward a nest. By altering plume characteristics (release rate, ambient flow, odor concentration), future studies could examine the relative importance of chemotactic and rheotactic mechanisms for the orientation of gravid females in a turbid environment. Finally, the combined role of visual, vocal and chemical signals in the spawning behaviour of the round goby could be investigated using realistic models, audio recordings and male urine samples (or artificial pheromones) as experimental stimuli.

From a conservation point view, the characterization of the round goby pheromonal system will be a significant asset to control the round goby population in the Laurentian Great Lakes and elsewhere. Future pheromone baited traps could be used to control the spread of this invader to new areas or locally to protect sensitive areas where native species spawn. Ultimately, the understanding of the round goby pheromonal system will improve our

understanding of the critical role that chemical signals play in orchestrating many animal interactions.

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APPENDICES

APPENDIX A: NUMBER AND DURATION OF URINATION EVENTS BY REPRODUCTIVE AND NON-REPRODUCTIVE MALES ROUND GOBY. 2008 AND 2007 DATA.

REPRODUCTIVE MALES 08

	No of UE				UE duration (in sec)							
Trial #	CtrlNRF	NRF	CtrlRF	RF	CtrlNRF		NRF		CtrlRF		RF	
					S	L	S	L	S	L	S	L
1	0	0	0	0	0	0	0	0	0	0	0	0
3	0	2	0	0	0	0	0	41	0	0	0	0
4	0	0	0	3	0	0	0	0	0	0	7	26
5	0	0	0	1	0	0	0	0	0	0	0	20
6	2	0	3	2	0	37	0	0	7	27	0	7
7	0	0	0	0	0	0	0	0	0	0	0	0
8	1	0	0	0	0	25	0	0	0	0	0	0
10	4	1	1	1	34	18	16	0	15	0	0	17
12	0	0	2	0	0	0	0	0	31	0	0	0
13	0	2	3	1	0	0	9	3	4	43	10	0
16	0	0	0	0	0	0	0	0	0	0	0	0
17	0	1	0	1	0	0	11	0	0	0	0	13
18	0	1	0	1	0	0	0	54	0	0	0	62
19	2	1	2	1	5	17	0	45	0	32	0	22
21	2	4	2	4	30	0	28	0	14	8	36	15
27	0	0	0	1	0	0	0	0	0	0	25	0
29	0	0	2	1	0	0	0	0	0	31	72	0
Sum	11	12	15	17	69	97	64	143	71	141	150	182
Mean	0.647	0.706	0.882	1	4	6	4	8	4	8	9	11
Std De	1.169	1.105	1.166	1.118	11	11	8	18	8	15	19	16
Std Err	0.284	0.268	0.283	0.271	3	3	2	4	2	4	5	4

NON-REPRODUCTIVE MALES 08

	No of UE				UE duration (in sec)							
Trial #	CtrlNRF	NRF	CtrlRF	RF	CtrlNRF		NRF		CtrlRF		RF	
					S	L	S	L	S	L	S	L
24	0	1	0	1	0	0	0	29	0	0	0	60
25	0	0	1	1	0	0	0	0	0	9	0	9
26	0	0	1	1	0	0	0	0	0	23	0	41
28	0	1	0	0	0	0	0	11	0	0	0	0
31	2	1	0	2	0	18	0	32	0	0	0	7
32	0	1	0	2	0	0	25	0	0	0	0	91
33	1	1	1	1	0	17	0	18	0	27	0	24
34	1	1	0	0	0	0	0	45	0	29	0	0
36	1	1	1	1	0	23	0	16	0	15	0	10
37	1	2	1	0	0	32	0	38	0	14	0	0
38	1	0	1	1	17	0	0	0	0	22	0	25
39	0	0	1	1	0	0	0	0	0	37	29	0
40	0	0	1	2	0	0	0	0	0	28	0	29
41	1	0	1	3	10	0	0	0	9	0	31	0
42	0	2	0	0	0	0	47	32	0	0	0	0
43	0	1	0	2	0	0	0	35	0	0	10	17
Total	8	12	9	18	27	90	72	256	9	204	70	313
Mean	0.5	0.75	0.563	1.125	2	6	5	16	1	13	4	20
Std De	0.632	0.683	0.512	0.885	5	11	13	17	2	13	10	26
Std Err	0.158	0.171	0.128	0.221	1	3	3	4	1	3	3	6

REPRODUCTIVE MALES 07

No of UE **UE duration (in sec)**

Trial #	CtrlNRF	NRF	CtrlRF	RF	CtrlNRF		NRF		CtrlRF		RF	
					S	L	S	L	S	L	S	L
1	0	0	0	1	0	0	0	0	0	0	10	0
9	1	0	0	3	5	0	0	0	0	0	9	25
10	0	1	0	1	0	0	0	21	0	0	5	0
12	0	0	2	0	0	0	0	0	2	19	0	0
17	0	1	1	1	0	0	0	31	0	40	0	21
18	0	2	0	0	0	0	5	8	0	0	0	0
19	2	0	0	0	0	39	0	0	0	0	0	0
21	1	0	0	1	0	0	0	19	0	0	0	12
22	0	0	0	1	0	0	0	0	0	0	0	16
24	3	0	1	2	3	39	0	0	1	0	10	0
27	0	5	0	0	0	0	6	18	0	0	0	0
30	0	2	0	0	0	0	4	0	0	0	0	0
34	0	0	0	2	0	0	0	0	0	0	1	14
37	1	0	0	1	0	15	0	0	0	0	0	14
Total	8	11	4	13	8	93	15	97	3	59	35	102
Mean	0.571	0.786	0.286	0.929	1	7	1	7	0	4	3	7
StdDEV	0.938	1.424	0.611	0.917	2	14	2	11	1	11	4	9
StdER	0.234	0.356	0.153	0.229	0	4	1	3	0	3	1	2

NON-REPRODUCTIVE MALES 07

	No of UE				UE duration (in sec)							
Trial #	CtrlNRF	NRF	CtrlRF	RF	CtrlNRF		NRF		CtrlRF		RF	
					S	L	S	L	S	L	S	L
35	1	1	1	1	0	9	0	15	0	20	0	10
43	0	2	1	3	0	0	3	0	5	0	10	0
44	0	0	4	4	0	0	0	0	5	0	4	0
47	0	3	1	1	0	0	18	0	4	0	10	0
48	1	1	1	1	10	0	0	5	10	0	8	0
total	2	7	8	10	10	9	21	20	24	20	32	10
Mean	0.4	1.4	1.6	2	2	2	4	4	5	4	6	2
StdDEV	0.548	1.14	1.342	1.414	4	4	8	7	4	9	4	4
StdER	0.245	0.51	0.6	0.632	2	2	3	3	2	4	2	2

APPENDIX B: STEP-BY-STEP PROTOCOL FOR PARTICLE IMAGE VELOCIMETRY ANALYSIS

PRE-PROCESSING:

-STEP 1: Extract a PIV video clip using FLASH DVD-RIPPER

FLASH DVD-RIPPER is used to extract video clips of chosen length from a DVD format to MPEG format.

- Insert a DVD
- Open DVD-ripper
- Click on OPEN DVD and select the Chapter of interest
- Make sure the option “MPEG” is selected on the main window of FLASH DVD-RIPPER (AVI format result in lower video quality)
- Select a folder location where to save the video clip using the “save as” bar at the bottom of the main window
- Click on the “convert” button to open the “Output Parameters” window
- On the chapter section, select “Select a clip” and click on the “select” button
- Use the cursor to select the “start point” and “end point” of the video clip of interest (note that FLASH DVD-RIPPER calculate the number of frames in the selected video clip). Click “OK”
- For better video quality, select the “MPEG 2” option in the “MPEG format” section
- In the “outpout video size” section, select “resize output video” and video size 720 x 480 (Best quality with the HITACHI video)
- Click OK
- FLASH DVD-RIPPER automatically extracts the selected video clip in the chosen folder. It usually takes 1min to extract a 1min video clip

-STEP 2: Create snapshots of the MPEG video clip using IMAGE VIDEO MACHINE

IMAGE VIDEO MACHINE is used to capture consecutive snapshots of a video clip at a chosen frequency. With a regular camera the maximum sampling frequency is 25 images/second. A high sampling frequency is necessary to perform PIV analysis.

- Click on the “Image ← Video” tab and use the input video field to open a MPEG PIV video clip.

- In the “Output option” section, select a picture format. The PNG offers the best picture quality.
- Choose 25 images per second
- Finally select an output folder and click “Extract Images”

-STEP 3: Subtract the picture background using IMAGEJ

Bright objects in the picture’s background result in unwanted correlations during the PIV analysis. The background of a picture can be erased using the “image calculator” function of IMAGEJ. This function can be automatically applied to a sequence of pictures called “stack”. However, because of memory limitation, this process can be applied only to stacks of 350 pictures at a time (14 s of video sampled at 25 pics/sec). Thus, the process must be repeated several times for PIV clips longer than 14s.

- In IMAGEJ choose File/Import/Image Sequence
- Open the first image extracted in STEP 2
- In the panel “sequence option”, enter the number of image (maximum 350) and the starting image (for instance 1) and click OK to created an images stack
- Now that the images stack is created, click on the menu Image/Stacks/ Z project
- Select “start slice” 1, “stop slice” 3 and “Projection type: Min intensity”. Click OK to create an image named MIN_[name of you file]
- Click on the menu Process/Image Calculator
- Select “image 1” the name of your image stack, “operation: subtract” and “Image 2” the image named MIN_[name of you file]. Click OK and OK again to process the entire images stack
- You get a new images stack without background. Save the images by clicking File/Save as/Image Sequence. Choose the PNG format for the best quality image
- If your PIV clip was longer than 14s (more than 350 images), repeat the same process until every images have been “cleaned”

PIV ANALYSIS WITH JPIV

The application JPIV can be downloaded at: http://www.jpiv.vennemann-online.de/. Follow the instruction on the website to install the application and set the library file

- STEP 1: JPIV settings

- In the Settings frame of JPIV, click on “General” and select the option “Consecutive”
- In the Settings frame of JPIV, Click on “Interrogation Window”
- In the “Multipass” field choose 1
- In the **interrogation window width** field write 64
- In the **interrogation window height** field write 64
- In the **search domain field width** write 32
- In the **search domain field height** write 32
- In the **horizontal vector spacing** field write 32
- In the **vertical vector spacing** field write 32
- In the “between passes” field select the options “Normalized median test”, “replace invalid vectors by median” and “3 x 3 smoothing”
- Do not change the other option on this page

- STEP 2: Run the PIV analysis

- Click on file/choose files and open the images created during the pre-processing (use the shift key to select a range of files)
- In the Files frame select the files that you just opened (use the shift key to select a range of files)
- Click on PIV/Run PIV image analysis. The save window opens
- In the save window choose the destination folder for the PIV vector files that JPIV will create. Name the vector file: [name of your file]_vector
- JPIV analyze the PIV images two by two. Depending of the number of images to analyze, this process can take from a few minute to a few hours.

- STEP 3: Filter the vector files

- In the Files frame select the vector files created in the previous step
- Click on Script/Batch_vector_filtering
- In the “edit parameters” window choose the following filtering parameters:

normMedTst 1

invIsolated 1

replByMed 1

rmInvalid	1
medFilt	0
medFiltAll	0
smooth	0
smoothAll	0

- Click Execute. In the save window, choose a destination folder and name the file: [name of your file]_vector_filtered

- STEP 4: Create a time-average vector file

- In the files frame, select the filtered files created in the previous step
- Click on Vector/Average vector field
- In the save window, choose the destination folder and name the file: [name of your file]_vector_filtered_averaged

- STEP 5: Use a color scale to display the flow velocity Vx

- In the settings frame, click on Preferences/Vector plot
- In the Background section, select the option “background picture” and select an image file to be used as a background for the PIV vector file
- Select the option “color coding” and in the field “data column” write 2

  (When creating a PIV vector file, JPIV sorts data into four columns: Columns #0 and #1 correspond to the x and y coordinates of each velocity vectors. Columns #2 and 3 correspond to the Vx and Vy components of each velocity vectors. A column #4 can be created for example when calculating vorticity)
- Set the maximum and the minimum values for the flow velocity (for example -3 and 8)
- In the Files frame, click on the time-average vector file created in step 4 to observe the final result of the PIV analysis.

- STEP 6: Create an image file from the PIV vector file

- In the Files frame, click on the time-average vector file created in step 4
- Place the cursor on the vector image and right-click

- Click on the option “export as a pixel image” to save the file as an image file that can then be displayed with any other software (i.e. MS Word, Excel, Powerpoint)

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