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FANNING BEHAVIOUR AND COMMUNICATION IN THE ROUND GOBY (*NEOGOBIUS MELANOSTOMUS*), AN INVASIVE FISH

By

Kristina Elizabeth Wantola

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2013

FANNING BEHAVIOUR AND COMMUNICATION IN THE ROUND GOBY

(*NEOGOBIUS MELANOSTOMUS*), AN INVASIVE FISH

By

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ABSTRACT

Determination of reproductive status in male round gobies can be challenging and the specifics of communication between males and females prior to spawning are unknown. First, I observed male fanning behaviour and compared this to both morphological and physiological measurements to determine if fanning is related to condition or reproductive status. My findings suggest that fins may be a useful measure of reproductive status and that fanning behaviour may be an important aspect of courtship potentially revealing parental abilities. I subsequently examined female responses to changes in flow in the presence of reproductive and juvenile male conditioned water using a y-maze flume experiment. My findings revealed that reproductive females prefer low flow conditions and reproductive conditioned water, whereas non-reproductive females prefer the opposite. This research represents the first investigation into the possible role of displacement fanning in this species and whether females may be using flow when evaluating potential mates.

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CHAPTER 1 – GENERAL INTRODUCTION

Olfaction in fish is highly developed and mediates many physiological and behavioural responses to chemical signals (Liley, 1982). Odours are thought to be similar to ornaments in that they can be used to enhance an individual's mating success by sending a strong signal to the opposite sex (Wyatt, 2003). For example, in the goldfish (*Carassius auratus*), females will release a preovulatory pheromone that synchronizes an increase in luteinizing hormone stimulating testicular synthesis and increases a male's fertility (Kobayashi *et. al.*, 2002). Pheromones are defined as an odour or mixture of odours that when released elicit a specific response in the receiver without prior learning or experience (Sorensen & Stacey, 2004). Pheromones can also include a variety of different odours including trail marking odours, and sex attractants (Corkum & Belanger, 2007). Pheromones may be further divided into two classes primer or releaser effects. Primer pheromones elicit a developmental and/or endocrinological response that might not be evident for a period of time (hours or days); however releaser effects elicit behavioural changes almost immediately (Stacey, 1983; Sorensen & Stacey, 2004). For example, in the rudd (*Scardinius erythrophthalmus*), females are thought to release a preovulatory pheromone, which has a priming effect on male hormones and sperm allocation (Stacey *et. al.*, 2012). In the round goby (*Neogobius melanostomus*), males increase gill ventilation rates when exposed to estrone (Belanger *et. al*., 2006), a precursor for 17β-estradiol an important sex steroid in females (Clelland & Peng, 2009). Similalry, in the zebrafish (*Brachydanio rerio*), females release a sex (signalling)

pheromone which elicits a motor response in males as they are attracted to the odour (Van Den Hurk & Lambert, 1983).

In fish sexual ornamentation can be quite diverse as some species exhibit sexual dimorphism throughout the year and others will develop additional ornaments only during the breeding season (Andersson, 1994). These ornaments can include changes in colouration (Wootton, 1976), development of additional structures (e.g. tubercles; Wedekind, 1992), or elongation of existing structures (e.g. fins; Basolo, 1990). Changes in colouration can include the intensification of sexual dichromatism or the development of a different nuptial colouration (Kodric-Brown, 1990). For example, the male stickleback develops a different nuptial colouration where the iris becomes blue and a blue tinge develops on the back in addition to red colouration on the mouth, throat, forebelly and opercular regions (Wootton, 1976). Similarly, in the bluegill (*Lepomis machrochirus*) males will develop complex breeding colouration, consisting of a blue head, red-orange breast and belly, and black pelvic fin (Kodric-Brown, 1998).

Many species of fish develop additional structures during the breeding season which they often loose when the season ends. For example, in the roach (*Rutilus rutilus*) males will develop breeding tubercles spread over the body prior to spawning and these will then fall off shortly after spawning occurs (Wedekind, 1992). Male minnows (*Phoxinus phoxinus*) will also develop breeding tubercles during the spawning season and these ornaments are suggested to play a role in the dominance hierarchy of this species (Jacob *et. al.*, 2009).

Many species of fish also show sexual dimorphism of fins usually with males developing larger and more elaborate fins (Turner, 1993). In the swordtail (*Xiphophorus helleri*), males exhibit long caudal extensions, the length of which is preferred by females (Basolo, 1990). Similarly, in the grass goby (*Zosterisessor ophiocephalus*) parental males will develop elongations of the fin rays on the second dorsal fin distinguishing them from parasitic males that may try to steal fertilizations (Torricelli *et. al.*, 2000). Six of the species within the genus *Neogobius* exhibit changes in their fins during the spawning season (Miller, 2003) illustrating the potential importance of fins and reproduction. Although the function for fin changes within the genus *Neogobius* is unclear, these changes have been well studied in other gobies. For example, in the common goby (*Rhinogobius brunneus*) males have a longer and more elaborate first dorsal fin which is strongly preferred by females (Suk & Choe, 2002). Similarly, in the green bubble goby (*Eviota prasina*) males develop longer dorsal fins than females and use these during courtship behaviours (Sekiya & Karino, 2004). Female green bubble gobies spend more time near courting male with longer rather than shorter dorsal fins (Sekiya & Karino, 2004).

Fins may be under sexual selection and have been suggested as honest indicators of quality in some species (Bakker & Mundwiler, 1999). Male quality can be expressed by the "evolution of costly, phenotypically variable traits whose expression reflects survivorship and vigor of males and hence their overall genetic quality" (Kodric-Brown & Brown, 1984). During mate choice females may use either putative benefit (e.g. nest defense ability) or a feature of behaviour or appearance that predicts benefits (e.g. large

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size meaning nest defense ability) as criteria for determining male quality (Dugatkin & FitzGerald, 1997). In the guppy (*Poecilia reticulata*), males develop larger tails, which are preferred by females, but larger tails reduce the male's swimming ability and increase his risk of predation (Karino *et. al.*, 2006). Thus, only higher quality males would be able to successfully advertise such traits (Zahavi, 1975). Similarly, in the threespine stickleback (*Gasterosteus aculeatus*), a correlation between relative pectoral fin size and the infection status of reproductive males has been reported (Bakker & Mundwiler, 1999).

Many of the above examples illustrate the importance of ornaments that are visually striking for mate attraction and assessment. However, visual cues are not always available because demersal fishes typically live in dark environments. Because communication is important for sexual selection via mate choice (Plath *et. al.*, 2004), acoustic, olfactory and mechanosensory signals become increasingly important under low visibility so that individuals can communicate with potential mates.

Mechanoreception plays an important role in the reproduction of fishes. In fish, the lateral line is a specialized mechanoreceptive organ made of a series of canals containing receptors (also known as neuromasts) which are specially adapted for aquatic life (Bleckmann, 1993). The lateral line network detects movement and vibrations in the water serving to locate both animate and inanimate objects that aid in behaviours such as feeding, predator avoidance and intraspecific communication (Blackmann, 1993). For example, Montgomery & Milton (1993) showed that the lateral line played an important role in the nocturnal feeding behaviour of the torrentfish (*Cheimarrichthys fosteri*). When

the lateral line system was blocked using chemical agents, fish were no longer able to locate or capture prey in the dark demonstrating the importance of the lateral line system in this fish (Montgomery & Milton, 1993). Many fish display vibrational behaviours during courtship, suggesting the lateral line may also be associated with reproduction (Bleckmann, 1993; Sargent *et. al.*, 1998). In the grass goby (*Zosterisessor ophiocephalus*), females display dorsal fin oscillations, which create small water vibrations and are thought to communicate spawning readiness to the male (Marchesan *et. al.*, 2000). Another example is in the himé salmon (*Oncorhynchus nerka*), where females display a vibrational "prespawning act" that then elicits spawning behaviour in the male (Satou *et. al.*, 1994a). This vibrational behaviour was found to be able to elicit normal male spawning even when visual cues were eliminated (Satou *et. al.*, 1994a). Researches further demonstrated that if the lateral line of males is blocked using chemicals that spawning behaviour does not take place even in the presence of the vibrational behaviour, suggesting that the lateral line is involved in communication between male and female for spawning synchronization (Satou *et. al.*, 1994b).

Role of Parental Care in Mate Choice

Male parental care is relatively common in fish with 50% of fish families that exhibit parental care exhibit "male only" parental care (Gross & Shine, 1981; Sargent, 1997). This type of care is defined as when the male provides all care to the offspring while still displaying elaborate ornamentations and competing for mates (Andersson, 1994). Male parental care is thought to be under a special form of sexual selection whereby males attract females to spawning territories allowing males to simultaneously

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care for offspring and court additional mates (Andersson, 1994). In this case, the need for sex role reversal is eliminated because parental care is not a resource over which females must compete (Williams, 1975). For example, in the threespine stickleback (*Gasterosteus aculeatus*), males will guard a nest during the spawning season and provide sole parental care (Wootton, 1976). Threespine stickleback males also develop bright nuptial colouration and court females (Wootton, 1976).

The operational sex ratio (OSR) plays an important role in sexual selection and parental care by changing the dynamics of a population and influencing which sex will be more competitive (Andersson, 1994). In the round goby, there is a highly male biased OSR of up to 6:1 in newly invaded sites so that males compete for females (Corkum *et. al.*, 2004). However, in native areas this ratio can change to 1:1 with males still competing for females. In some species, the OSR of a population can change over time and within a single breeding season. For example, in the two-spotted goby (*Gobiusculus flavescens*), there is a change from a male biased OSR to a female biased OSR during the breeding season as males become rare (Svensson *et. al.*, 2008). Females become the more competitive sex towards the end of the breeding season, developing ornamental colouration and competing for males (Svensson *et. al.*, 2008).

Females of species with paternal care may select males based on their parental abilities using cues such as courtship behaviour (Knapp & Kovach, 1991) or intensity of body colouration (Milinski & Bakker, 1990). Hoelzer (1989) suggested that females may choose potential mates based on their parental abilities. Fanning behaviour is an important parental care behaviour because it provides oxygen to developing embryos and

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has been shown to play a key role in egg survival (Karino & Arai, 2006). Lindström *et. al*. (2006) showed that sand goby (*Pomatoschistus minutus*) males may use parental care behaviours (e.g. fanning) as an important mechanism for female mate attraction. Displacement fanning (fanning behaviour that begins before spawning occurs) has been suggested as an honest indicator of male parental care abilities (Sevenster, 1961; Bakker & Mundwiler, 1999).

Parental care behaviours are energetically demanding (Jarvi-Laturi *et. al.*, 2008) and therefore males of lower quality would be less able to provide quality care to offspring (Unger & Sargent, 1988). Males of good quality may signal their condition to females through their behavioural display rates (Sargent, 1997). For example, sand goby males increase their fanning in the presence of females, suggesting the use of fanning as a courtship behaviour (Pampoulie *et. al.*, 2004). Females are expected to prefer males that reliably advertise their parental capabilities because females are not able to compensate for lower levels of paternal investment (Stivers & Alonzo, 2009).

Courtship Behaviour in the Round Goby

The round goby is a small benthic fish species, which is native to the Ponto-Caspian region of Eastern Europe (Charlebois *et. al.*, 1997). The round goby was introduced into the Laurentian Great Lakes in the 1990's via ballast water and rapidly spread to all five of the Laurentian Great Lakes (Jude *et. al.*, 1992; Charlebois *et. al*., 2001). Round gobies spawn multiple times within a single reproductive season (May-October) and have high fecundity allowing them to outcompete native species (Corkum *et. al.*, 1998; MacInnis & Corkum, 2000). During the reproductive season, reproductive

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male round gobies will excavate a nest, usually under a rock, or more typically occupy a shelter from which they will attract reproductive females (Charlebois *et. al.*, 2001).

Gammon *et. al.* (2005) showed that reproductive female round gobies are attracted to the conditioned water of reproductive males suggesting that males use sex pheromones. In the field a single male attracts up to 15 females in a single nest (MacInnis & Corkum, 2000). Reproductive males will guard their nest during the reproductive season and perform various courtship displays including displacement fanning (Meunier *et. al.*, 2009). Additionally, males are able to produce a strong directed current of water pumping potential odours and waste out of the nest (Meunier, 2009). Female round gobies often orient themselves perpendicular to the displaying male in the nest (Meunier *et. al.*, 2009), suggesting the use of mechanosensory signals in addition to pheromones for mate attraction. The round goby possesses superficial neuromasts throughout the body (Charlebois *et. al.*, 1997) which are more sensitive than other types of enclosed neuromasts (Jude *et. al.*, 1995). Thus, round gobies may be particularly sensitive to changes in flow created by male fanning behaviours.

Objectives

The specifics of round goby reproduction were recently characterized by Meunier *et. al.* (2009); however, specific mechanisms used in communication between males and females during the pre-spawning period is yet unknown. Additionally, determination of reproductive status in male round gobies can be challenging because two males with the same gonadosomatic index (GSI) levels may have different androgen levels (Corkum & Cogliati, 2013). The exact method by which female round gobies assess potential mates

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is also unknown. Nest-guarding males exhibit fanning behaviours prior to egg deposition (displacement fanning) and it has been speculated that fanning may play a role in pheromone dispersal (Meunier *et. al*., 2009) or as an honest indicator of male parental abilities (Sevenster, 1961; Bakker & Mundwiler, 1999).

The first objective of my thesis was to determine if displacement fanning behaviour of nest-guarding male round gobies was related to the condition and/or reproductive state of the male. Females are thought to examine signals indicating male quality when assessing a potential mate (Sevenster, 1961; Zahavi, 1975; Hoelzer, 1989; Lindström, 2006). Since advertisement of signals is costly (Zahavi, 1975), I hypothesized that reproductive males should relay information about their quality to potential mates prior to spawning by fanning. If male fanning relays information about male quality, I predict that fanning should be related to the condition and reproductive state of the male. I placed males exhibiting secondary sexual characteristics into tanks containing several gravid females and recorded fanning behaviour. I subsequently dissected males to measure morphological (e.g. fin size, condition factor) and physiological traits (e.g. gonadosomatic index, 11-ketotestosterone levels) to confirm the reproductive status of the fish. I then compared different aspects of both pectoral and caudal fanning behaviours (rate, bout duration, bout frequency) with the morphological and physiological measurements to determine if fanning behaviour reflected male quality.

Male round goby caudal fanning behaviour creates a strong directed current of water out of the nest and females often orient themselves perpendicular to the displaying male (Meunier *et. al.*, 2009). This orientation by the female suggests that females detect

flow patterns created by male fanning in addition to olfactory signals. Previous research

by Gammon *et. al.* (2005) demonstrated a reproductive female preference for the

conditioned water of reproductive males. Thus, the second objective of my thesis was to

test the response of females to changes in flow in the presence of conditioned water from

reproductive and juvenile male round gobies. I used a Y-maze experimental flume set-up,

similar to that of a Grice box (Kolb *et. al.*, 1983), (with six different treatment

combinations to test female preference for 1) high and low flow conditions and 2)

conditioned water of reproductive and juvenile male round gobies. Because high quality

males are thought to pump water and putative pheromones out of the nest, I predicted that

reproductive females would prefer high flow conditions and reproductive male

conditioned water. An integrative approach was used to elucidate patterns in female mate

choice using behaviour, GSI and estrogen (17β-estradiol). GSI and estrogen were used as

measures of female reproductive status because a female's state can influence

behavioural patterns (Ramsey *et. al.*, 2011).

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CHAPTER 2 – DISPLCEMENT FANNING AS AN INDICATOR OF REPRODUCTIVE STATUS IN NEST-HOLDING MALE ROUND GOBIES (*NEOGOBIUS MELANOSTOMUS*)

INTRODUCTION

In many species, females are often choosy, favouring males that exhibit parental care (e.g. fanning behaviours; Hoelzer, 1989; Andersson, 1994; Dugatkin & FitzGerald, 1997; Lindström, 2006) because these behaviours are an important component of parental care and play a key role in egg survival (Karino & Arai, 2006) by providing eggs with oxygen during development (Meunier *et. al.*, 2009). Egg fanning behaviours are common in many fishes, including the threespine stickleback (*Gasterosteus aculeatus*; Wootton, 1976), orange chromide (*Etroplus maculatus*; Zoran & Ward, 1983), cinnamon clownfish (Amphiprion melanopus; Green & McCormick, 2004), as well as may other fish that exhibit parental care. There are however, few studies examine the importance of fanning that begins before the presence of eggs. Displacement fanning, fanning behaviour that begins before eggs are deposited in the nest, is thought to be an honest indicator of male parental abilities (Sevenster, 1961; Bakker & Mundwiler, 1999); i.e., males send honest signals to females about their energy reserves through courtship intensity (Sargent, 1997). Male displacement fanning increases in the presence of females (Sevenster, 1961; Pampoulie *et. al.*, 2004) and can decrease with the prolonged absence of females (Sevenster, 1961). When fanning has decreased, presenting the male with a female can immediately revive fanning activity (Sevenster, 1961) illustrating the important of female presence for displacement fanning activities and suggesting that females may be evaluating males through fanning behaviour.

The round goby (*Neogobius melanostomus*) is an invasive fish that has been introduced into the Laurentian Great Lakes (Jude *et al.*, 1992). Males exhibit a variety of discernible reproductive behaviours (Meunier *et al.*, 2009); however, it is surprisingly difficult to identify a reproductive male with certainty. Current measures of reproductive status in males include black nuptial colouration, elevated 11-ketotestosterone (11-KT) and gonadosomatic index (GSI) (Marentette & Corkum, 2008; Marentette *et al.*, 2009). Reproductive male round gobies develop black nuptial colouration during the spawning season, but not all black males are reproductively active. For example, males exhibiting aggressive behaviours may also develop this black colouration yet have minimal gonadal growth and do not guard a nest (Corkum, personal observation). Similarly, 11-KT, a primary fish androgen associated with secondary sexual characteristics (Borg, 1994), may also be an unreliable measure of reproductive status. Levels of 11-KT in the blood are highly variable among individuals during the reproductive season and can even vary throughout the day, making this a seemingly unreliable measure of reproductive status (Sisneros *et al.*, 2004; Lorenzi *et al.*, 2008). Likewise, GSI may be highly variable among individuals during courtship (Sisneros *et al.*, 2004; Zeyl, 2012). A GSI value of 1.3% is typically used to identify a reproductive male round goby (Belanger *et al.*, 2007). Since fanning behaviour prior to egg deposition may be important for courtship in the round goby (Meunier *et al.*, 2009), this behaviour also may provide an indication of male reproductive status.

In this study, I examined the potential for displacement fanning to be used as a predictor of reproductive status in the round goby. I began by determining which trait

best distinguished between nest-holding and juvenile (non-reproductive) male round gobies. Subsequently, I examined the relationships among fanning, morphological (fin size) and physiological (11-KT & GSI) traits of nest-holding males. Females are thought to evaluate males based on their quality, which can be expressed as either a putative benefit (e.g. nest defence ability) or as a feature of behaviour or appearance that can predict benefits (e.g. large size indicating nest defence ability) (Dugatkin & FitzGerald, 1997) and because advertisement of such signals is costly (Sevenster, 1961; Zahavi, 1975; Hoelzer, 1989; Lindström, 2006), I hypothesized that males in a reproductive state should relay information about their quality to potential mates before spawning. If males fan to relay information about their quality, I predicted that fanning should be related to the condition and reproductive state of the male.

MATERIALS AND METHODS

Collection of animals

Round gobies were collected during late spring and summer of 2011 and 2012 by angling and seining along the Detroit River (42^o18'N, 83^o04'W) and Lake Erie (42^o15'N, 81^o54'W). Also, PVC tube (7.5 cm diameter, 20 cm length, 3 cm diameter opening) and square box (15 cm x 15 cm x 7 cm) nests were deployed at Erieau, Ontario, Canada, located on the northern shore of Lake Erie, and retrieved every 2 weeks to capture nest-holding males (cf. Yavno & Corkum, 2011). The fish were transported in coolers (2 hours), containing aerated lake water and shelters to the Animal Care Facility at the University of Windsor. To track individual fish, individuals were tagged using visual implant elastomer (VIE) tags (2012 only; Northwest Marine Technology) before the fish

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were placed into 90 L flow through communal tanks, containing approximately 20 fish. Communal tanks contained both males and females at a ratio of approximately 2:1. Fish were kept at a constant 16L: 8D light cycle at 18° C to simulate natural conditions during the spawning season (Meunier *et al.*, 2009). All tanks were lined with aquarium gravel and contained shelters. Fish were fed daily with excess amounts of Nutrafin ® flakes and remaining food was removed every 2-3 days as needed (Jones & Reynolds, 1999). All fish were housed and treated in accordance with the animal care protocols outlined by the Animal Care Committee at the University of Windsor and the Canadian Council on Animal Care.

Morphometric measurements

Male round gobies were kept in communal tanks until they began to fan. In 2011, individual males were immediately moved into a separate 20 L flow through tank once fanning began. In each tank, there was one male along with 3-4 females to enhance fanning. Occasionally, non-reproductive females (females not displaying prominent orange coloured papilla or distended belly) were added to the tank if there were insufficient numbers of reproductive females. In 2012, males were not removed from communal tanks as the VIE tags allowed us to distinguish among males within a single tank. If spawning occurred within the shelter then the males were recorded until the eggs had hatched or were eaten however, these data were excluded from the present study.

Once fanning was no longer observed, males were euthanized using clove oil (100 mg/L; Anderson *et. al.*, 1997), so that morphological traits could be measured. Fish were weighed to the nearest 0.01g using a digital balance (Ohaus Adventure Pro AV412C).

Head width and total length were measured in mm. Fulton's condition factor (K) was calculated for each male as weight/length3 (Ricker, 1975). Because fins play an important role in both fanning and courtship behaviours (Immler *et al.*, 2004; Meunier et al., 2009), surface areas of fins were taken from photographs obtained using a digital camera (Canon Powershot A430) and measured using ImageJ software (e.g. Abramoff *et al.*, 2004; http://rsb.info.nih.gov/ij/). All fins (1st dorsal, 2nd dorsal, fused pelvic, right pectoral, anal and caudal) were clipped and spread out on a piece of plasticized graph paper for these measurements. Graph paper was used to standardize each photo and calibrate the ImageJ software for consistency.

Physiological measurements

Samples were collected from the caudal vein using heparinized micro-capillary tubes to measure plasma levels of 11-KT following the procedures of Katare *et al.* (2011). Samples were always collected in the morning (09:00-11:00 h EDT) to account for diel changes in hormone concentrations (Lorenzi *et. al.*, 2008). Each blood sample was transferred to a 1.5 ml Eppendorf tube and centrifuged at 13,000 RPM for 10 min to separate the blood plasma from the rest of the sample. The plasma (ranging from 10-50 uL) was then removed using a micropipette and stored at -80°C until analyzed using an enzyme linked immunosorbent assay (ELISA; e.g. Möstl & Palme, 2010) for 11-KT levels. Plasma from individuals was thawed at room temperature and diluted 1:10 with assay buffer before application to the plate which had been coated with protein A (sticky plate; Appendix A). Individuals were randomly assigned in duplicate to a plate along with a standard sample of known concentration. This last sample was used to create a

standard curve to which the other samples were compared in order to calculate concentration of 11-KT within each of the samples.

Each male was dissected, gonads removed and weighed to the nearest 0.01 g. GSI values were determined using total gonad mass (testes + seminal vesicles) divided by the total mass multiplied by 100.

Video analysis of fanning behaviour

Male fanning activity was recorded for a minimum of 2 h using a video camera (Hitachi VKC-370) and DVD recorder (Sony RDR-GX330). Recordings were started at 09:00 h and continued throughout the day if the male continued to fan. Occasionally males began to fan later in the day so that some recordings were obtained from 15:00 to 20:00 h. Recordings continued over several days, provided the male continued to fan. All recordings of fanning behaviour were done in the presence of females for both communal and individual tanks.

All videos were analyzed for the following behaviours: 1) fanning rate (number of fin beats per minute, bpm), 2) fanning bout duration (length of continuous fin movement in seconds) and 3) frequency of fanning bouts (number of fanning bouts per hour, bph) for both pectoral and caudal fanning (cf. Östlund & Ahnesjö, 1998). Behaviours were measured by one individual (KW) to reduce observer error. To calculate fanning rate, three randomly generated times were selected for each hour of video. A mean value for fanning rate was determined for each individual male and used for subsequent statistical analyses. Fanning rate was calculated by dividing the number of continuous fin beats (both left and right fins for pectoral fanning and caudal fin for caudal fanning) by the

duration of the fanning bout. Fanning bouts nearest one of the designated time points

(within a maximum of 10 min) was determined visually. If two fanning bouts were

equally distant from the randomly generated time point, a coin toss was used to select the

bout to be examined. Fanning frequencies were calculated for either the 1st or 2nd 30 min

period (determined by coin toss) for each hour of video and doubled to estimate the

number of bouts per hour.

RESULTS

Determination of male reproductive status

The mean (±SE) GSI values for reproductive (1.59 ± 0.09) and non-reproductive

(0.19 ± 0.01) males differed significantly ($p < 0.01$). The GSI values for all nest guarding

males ($N = 37$) showed a wide range of values ($0.87 - 2.99\%$). Interestingly, 28% of

these males fell below the 1.3% cut-off commonly used to determine reproductive status

(Figure 2.1). I considered males with a GSI value $> 0.85\%$ to be reproductive and those

males with a GSI value of $< 0.45\%$ to be non-reproductive.

Morphological distinction between reproductive and non-reproductive males

A discriminant function analysis was used to determine which external

morphometric (head width, fin surface area) traits best delineated groups of reproductive

and non-reproductive males captured from the Detroit River and Lake Erie. The groups

were allocated on the basis of GSI values (see above). Variables best able to separate the

two groups of males were the surface areas of fused pelvic ($F_{(1,\ 103)} = 9.47$, $p = 0.003$) and

caudal ($F_{(1,\ 103)} = 8.87$, $p = 0.004$) fins. The discriminant analysis correctly classified 73

of the 74 cases for non-reproductive males (99% correct) and 29 of the 37 cases for

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reproductive males (78% correct). When combined the total number of cases correctly

identified was 102 out of a possible 111 (92%) cases (Figure 2.2). Reproductive males

have proportionally larger surface areas for the fused pelvic and caudal fins than do non-

reproductive males (Figure 2.3).

Fanning behaviour of nest-holding males

Fanning rates, frequencies and bout durations of nest-holding males were

compared for pectoral and caudal fanning (Figure 2.4). Caudal fanning rate (144.2 ± 4.13

bpm) was significantly higher (N = 24, p < 0.001) than pectoral fanning rate (95.25 ±

1.48 bpm) for individual males. Average caudal fanning rate ranged from 93 – 305 bpm;

average pectoral fanning rate ranged from 46 – 170 bpm. Pectoral fanning (24.53 ± 1.81

bph) was more frequent (N = 24, p = 0.037) than caudal fanning (14.15 ± 1.54 bph). The

length of pectoral (5.64 ± 0.38 s) and caudal (5.96 ± 0.37 s) fanning bouts were similar

(N = 24, p = 0.22).

Males that fanned more frequently with their pectoral fins also had longer pectoral

fanning bouts (N = 31, r = 0.45, p = 0.012). Males with longer caudal fanning bouts had a

lower average caudal fanning rate (N = 25, r = -0.48, p = 0.015) and lower maximum

caudal fanning rate (N = 25, r = -0.62, p = 0.001).

*Does fanning behaviour reflect the condition or reproductive status of nest-holding
males?*

When 11-KT was compared with fanning rate, there was a significant positive

relationship with maximum pectoral fanning rate (N = 24, r = 0.42, p = 0.041) (Figure

2.5); i.e., males with higher 11-KT levels exhibited a higher maximum pectoral fanning

rate. Other fanning metrics were unrelated to either 11-KT or GSI values. Fulton's condition factor was calculated for individual males. Those males that exhibited a higher maximum pectoral fanning rate exhibited a lower condition factor (N = 31, r = -0.428, p = 0.016) (Figure 2.6a). Moreover, males with a higher maximum pectoral fanning rate had a larger 1st dorsal fin (N = 31, r = 0.392, p = 0.029) (Figure 2.6b).

DISCUSSION

Determination of male reproductive status in the round goby can be complicated because the black nuptial colouration exhibited by reproductive males also can be exhibited by non-reproductive aggressive males. GSI, a commonly used index of reproductive status, can yield misleading results on reproductive status as two males with the same GSI may have different androgen levels (Corkum & Cogliati, 2013). Similarly, the androgen, 11-KT, may not be a good measure of reproductive status because values are highly variable within and among individuals during the reproductive season (Sisneros *et al.*, 2004) as well as over the course of a day (Lorenzi *et al.*, 2008). Morphological changes in the fins during the spawning season of three closely related gobiid species (*Neogobius caspius, Neogobius fluviatilis & Neogobius melanostomus*; Neilson & Stepien, 2009) suggest that fin traits play a role in courtship and reflect reproductive status (Miller, 2003). Fanning behaviours, which are essential for egg maintenance, may also be associated with courtship as evidence suggests a relationship between fanning and sexual activity (Sevenster, 1961). In general, morphological traits and behavioural signals are likely sexually selected (Sekiya & Karino, 2004; Lindström *et al.*, 2006).

Nest-holding male round gobies exhibited a wide range of GSI values, which fell below 1.3% cut-off value typically used to identify reproductive male round gobies (Belanger *et al.*, 2007). Because male gobies may be in different stages of development as they prepare for spawning, the use of GSI values may not always reflect reproductive status (Zeyl, 2012). Histological analyses performed by Zeyl (2012) showed that a GSI value of 1% distinguished between those males with and without sperm; however, distinct stages of spermatogenesis were poorly distinguished. Similarly in the African cichlid (*Astatotilapia burtoni*), the GSI value was not able to predict the number of mature sperm present in the testes (Maruska & Fernald, 2011), illustrating the importance of using multiple characteristics when determining reproductive status.

Results of the discriminant function analysis revealed that both pelvic and caudal fin surface areas separated groups of reproductive and non-reproductive males. This is consistent with the view that larger more elaborate fins could be under sexual selection as an honest indicator of parental care and/or male quality (Turner, 1993; Bakker & Mundwiler, 1999). Round gobies have a fused pelvic fin and during courtship they lift their bodies using this fin similar to what was described by Reynolds and Jones (1999) in the common goby (*Pomatoschistus microps*). This lifting behaviour may aid in protection of the nest by making the guarding male seem larger and better able to defend the nest against intruders since larger males often win in male-male competitions (Magnhagen & Kvarnemo, 1989; Forsgren, 1997). Lifting behaviour may also enable the male to better detect predators through chemical signals as seen in the Iowa darter (*Etheostoma exile*)

(Wisenden *et al.*, 1995). Similarly, the illusion of being larger could benefit the male

directly as females often prefer to mate with larger males (Lindström, 1992).

Nest-guarding male round gobies fan using their caudal fin, creating a strong

directed current out of the nest in contrast to pectoral fanning which creates a small

inward current (Meunier, 2009). The outward water current created by fanning is thought

to aid in the dispersal of pheromones that ultimately lure females to nests (Gammon *et

al.*, 2005). A male with a larger caudal fin should be able to push water farther and faster

(Bakker & Mundwiler, 1999) better dispersing pheromones and potentially attracting

more mates. Therefore, the elongation of the caudal fin, although potentially costly as

with any ornament (Zahavi, 1975; Basolo, 1990), could benefit the male in terms of

reproductive ability.

Pectoral fanning was observed to be much more common and rates were

significantly slower than caudal fanning (95 ±1.5 bpm and 144 ± 4 bpm, respectively).

There is likely a trade-off between the rate at which a fish can fan pectorally and the

length of time spent fanning. Fanning frequency is likely more important than fanning

rate in maintaining a consistent oxygen level for developing eggs (Östlund & Ahnesjö,

1998). Lindström *et al*. (2006) suggested that pectoral fanning plays a role in courtship

since it could be advertising the males' parental ability. Fanning to advertise parental

ability is consistent with a study by Karino and Arai (2006), where the time spent

pectoral fanning was the primary determinant of egg survival in the green bubble goby

(*Eviota prasina*). Caudal fanning rates were significantly faster than pectoral fanning

rates in this study and this increase in the caudal fanning rate should allow the male to pump more water out of the nest removing debris and other unwanted materials.

Males that fanned more frequently with their pectoral fins also fanned for longer periods of time: i.e. there was a positive relationship between fanning frequency and fanning bout duration ($p = 0.012$). Males that fan eggs more frequently, but for shorter periods of time, have a higher hatching success (Östlund & Ahnesjö, 1998). In contrast, males that fan more frequently but for longer periods of time have a lower hatching success (von Hippel, 2000). Nevertheless, males that expended more energy by fanning longer and more frequently still benefited, because these males were able to get females to spawn more easily allowing males to have more spawning events in a season (von Hippel, 2000).

Little is known about the fate of male round gobies at the end of the reproductive season. Parental care greatly decreases the condition of an individual because foraging by nest-holding males is limited (Miller, 1984). Marentette *et al.* (2012) recaptured a small number of males known to be nest guarding in the previous season, suggesting some males may overwinter. If males are only able to reproduce once, males in better condition that fan more vigorously (in terms of frequent bouts and duration) should be able to lure more females into their nest, resulting in increased reproductive success over other nest-holding males in poor condition.

Maximum pectoral fanning rate was negatively associated with condition factor and positively associated with the surface area of the 1st dorsal fin. An increase in the pectoral fanning rate has been associated with an increase in weight loss and therefore a

decrease in condition of nest-guarding male sand gobies (*Pomatoschistus minutus*) (Jarvi-Laturi *et al.*, 2008). Pampoulie *et al.* (2004) showed that males increase fanning in the presence of females, and Rohwer (1978) suggested that males may deceive females about egg presence in nests through fanning. Several hypotheses suggest why females may prefer to spawn with males whose nest contains eggs, including female copying (Jamieson, 1995) and reduction of filial cannibalism (Rohwer, 1978; Forsgren *et al.,* 1996). Accordingly, males in lower condition may increase their fanning to either attract females or to deceive them about the probability of eggs present in the nest. This strategy could explain the negative relationship that was observed between maximum pectoral fanning rate and the condition factor of nest guarding males.

The positive relationship between the maximum pectoral fanning rate and the surface area of the 1st dorsal fin may relate to male reproductive investment (Immler *et al.*, 2004). Parental males display courtship behaviours (fanning and nest guarding) and secondary sexual ornaments such as fin elongation (Sevenster, 1961; Turner, 1993; Immler *et al*., 2004). Breeding males of the round goby and related species (*N. caspius and N. fluviatilis*; Nielson & Stepien, 2009) have similar secondary sexual ornaments (Miller, 2003). The outer edge of the dorsal fins of all three species is white or yellow (Miller, 2003) and note that this light edge on the dorsal fin mimics the colour of developing eggs (Corkum pers. comm.). The edge of the dorsal fins and eggs are seen in sharp contrast to the dark interior of the nest and therefore may be used in conjunction with fanning to signal presence of eggs to females.

The maximum pectoral fanning rate was positively correlated with plasma levels of 11-KT (p = 0.041). This relationship is consistent with studies indicating that increases in 11-KT are related to increases in reproductive behaviour (Borg & Mayer, 1995). Magee *et al.* (2006) found that male bluegill sunfish (*Lepomis macrochirus*) that nested multiple times in a season had higher levels of 11-KT and were in better condition than were males that nested only once. Thus, male gobies exhibiting higher levels of 11-KT could advertise their parental quality by fanning at a higher rate.

Fanning behaviour is an important aspect of parental care in many fish, but begins well before spawning occurs, suggesting that it may be a useful measure of reproductive status. The maximum pectoral fanning rate of male round gobies was found to be significantly related to 11-KT levels as well as condition factor. These results suggest that fanning behaviour may be relaying important information about the male to potential mates about the males' condition and parental quality (Sevenster, 1961; Sargent 1997). Males of lower condition may be using fanning to deceive females about their quality in order to gain more spawning events (Rohwer, 1978). Fins themselves also are important for fanning and courtship behaviour as fins can change during the breeding season, suggesting that fins may also be used as ornaments for mate attraction (Miller, 2003). My study revealed that the surface areas of both the fused pelvic fin and caudal fin were able to successfully discriminate between reproductive and non-reproductive males. This indicates a potentially useful non-invasive measure for identifying reproductive males. Future research into the actual costs associated with displacement fanning in the round goby and measures of responses by females to various changes in male fanning behaviour

may provide further insight into whether females are using this behaviour to assess mate

quality.

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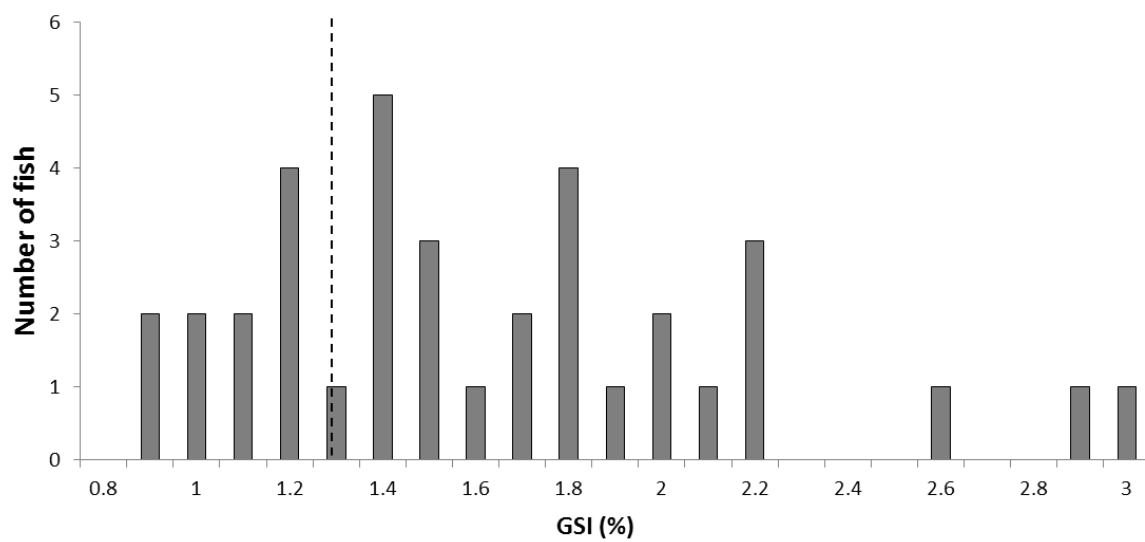
Figure 2.1 Histogram illustrating the range of GSI values obtained from dissection of a nest holding males (n = 36). The dashed line illustrates the 1.3% cut-off commonly used to distinguish between reproductive and non-reproductive
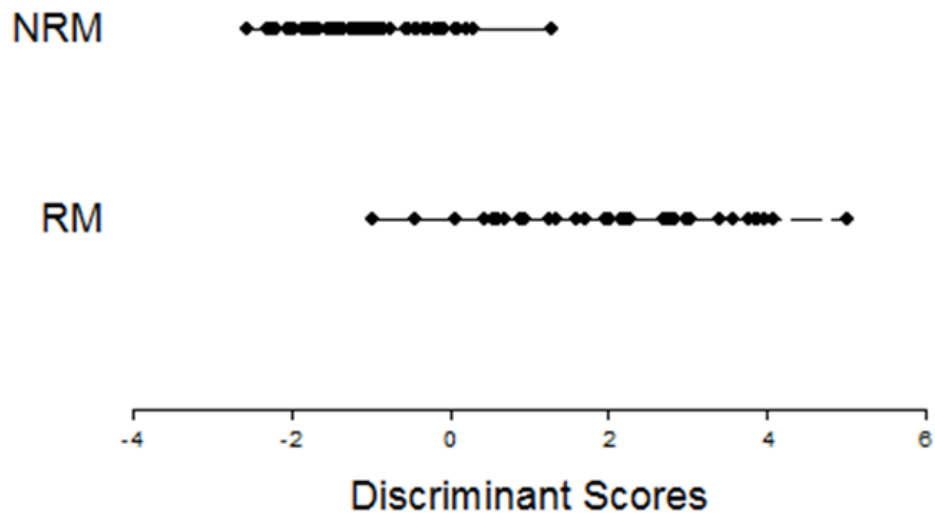
Figure 2.2 Discriminant scores for reproductive and non-reproductive male round gobies. The circles represent the discriminant scores for individuals of each group along the first discriminant axis.
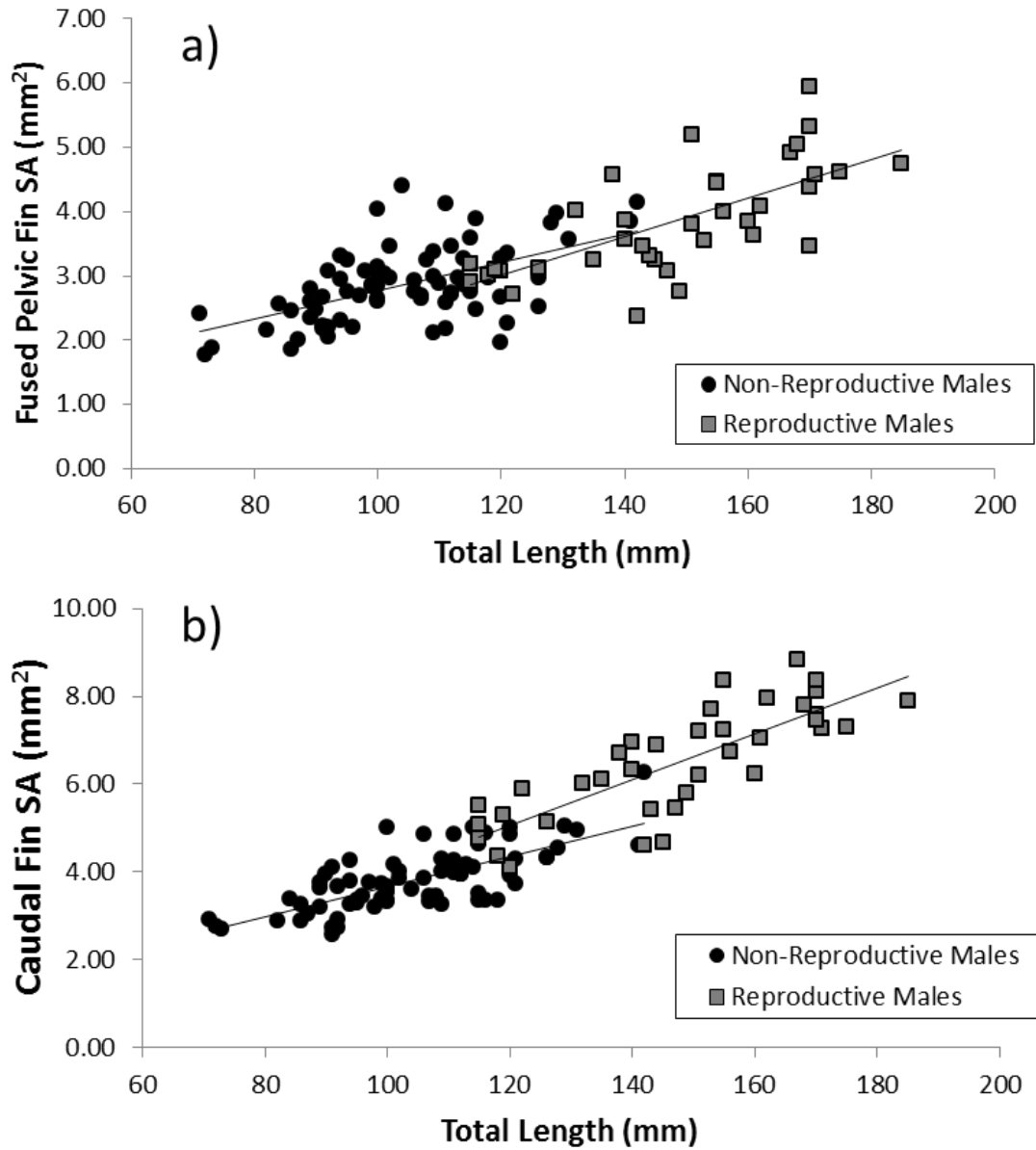
Figure 2.3 Correlation between a) fused pelvic and b) caudal fin surface areas and body size (total length) for reproductive (GSI > 0.85%) and non-reproductive (GSI < 0.045%) males.
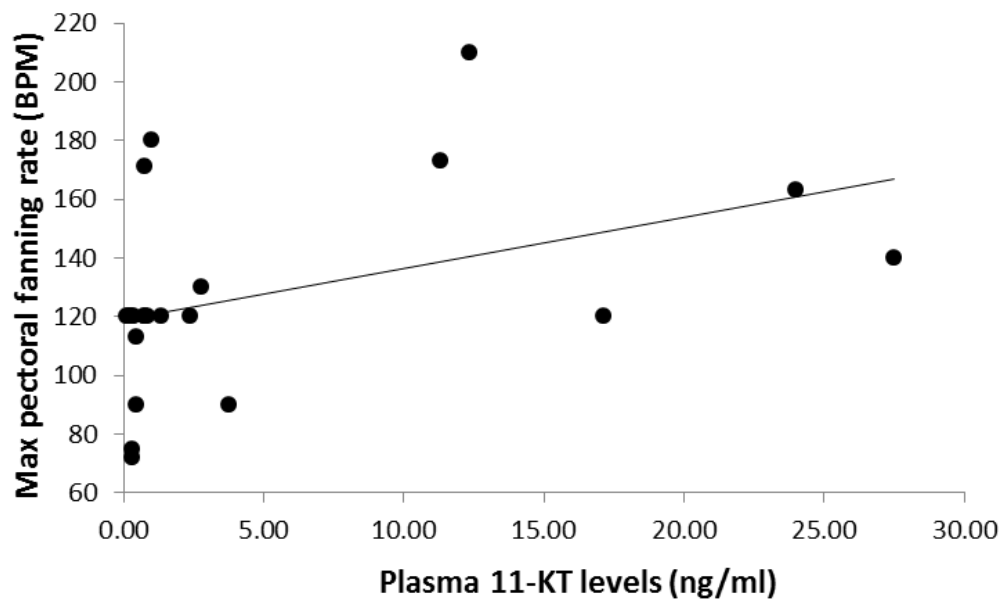
Figure 2.4 Correlation between maximum pectoral fanning rate and plasma 11-ketotestosterone level for each nest holding male (n = 24, r = 0.42, p = 0.041).
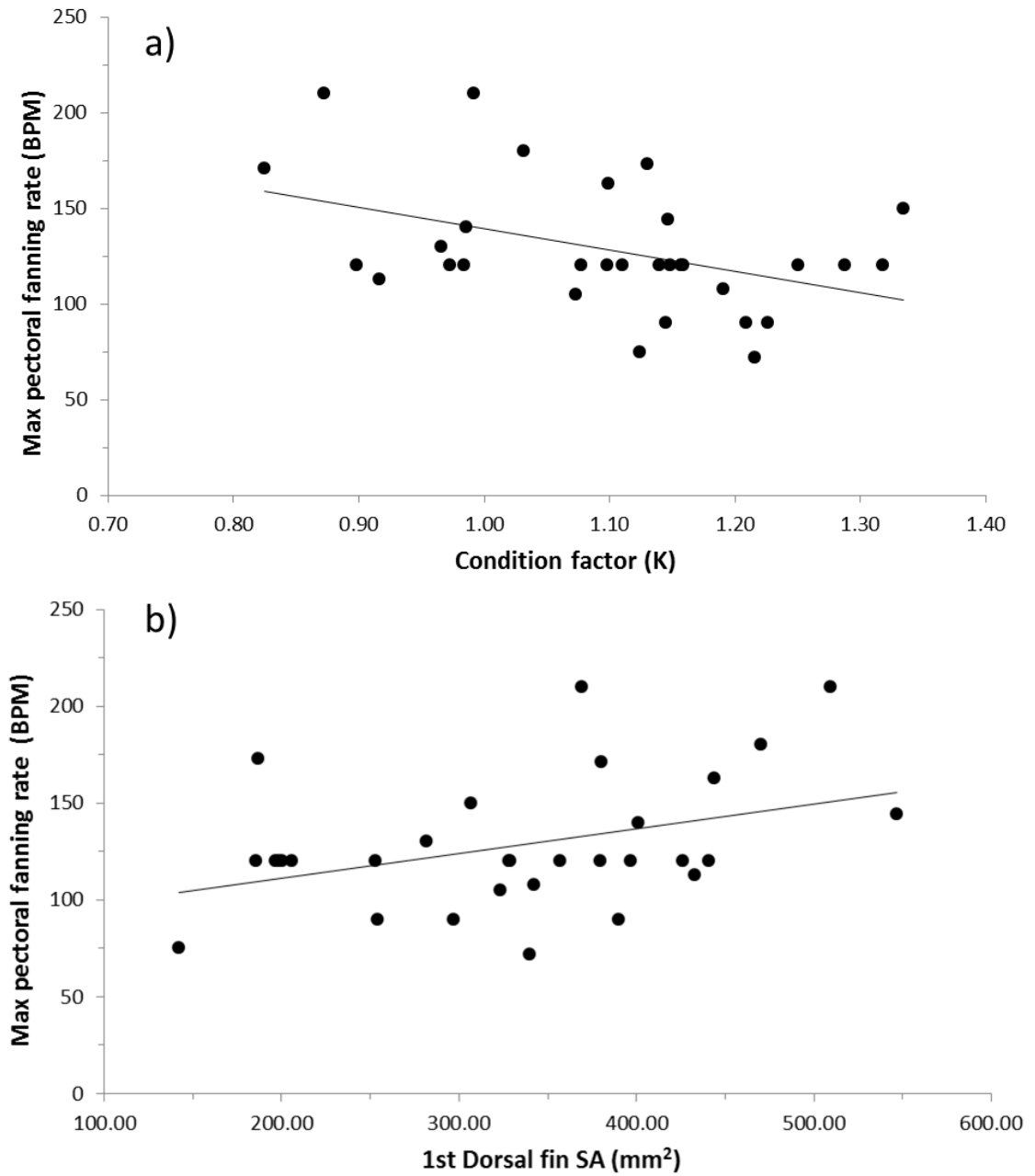
Figure 2.5 Correlation between a) the maximum pectoral fanning rate of a nest holding male and Fulton's condition factor (calculated as weight x 10^5/ length^3) (n = 31, r = -0.43, p = 0.016) and b) the maximum pectoral fanning rate of a nest holding male and the surface area of the 1st dorsal fin (corrected for size of the fish by dividing surface area by total length) (n = 31, r = 0.39; p = 0.029).

CHAPTER 3 – AN INTEGRATIVE APPROACH TO EXAMINE THE MECHANISMS BEHIND MATE CHOICE IN ROUND GOBIES, *NEOGOBIUS MELANOSTOMUS*

INTRODUCTION

Mate choice is a behaviour exhibited by an individual of one sex that increases the likelihood of it being selected to mate with certain members of the opposite sex (Andersson, 1994). Mate choice is usually based on either an immediate benefit such as nest quality and parental care or on a delayed benefit such as the fitness of offspring (Tuner, 1993). Potential interactions between breeding partners depend on an individual's ability to extract the information required from its surrounding environment (Moore & Crimaldi, 2004). However, courtship and mate choice are challenging for aquatic organisms when turbid environments limit the use of visual signals (Corkum & Belanger, 2007).

The olfactory system of fishes is highly developed, influencing both physiological and behavioural responses to both sexual and non-sexual chemical signals (Liley, 1982). Behavioural and physiological changes that can be elicited by sex pheromones are important for reproduction because these signals which initiate a response, often travel long distances (Liley, 1982) and are detectable at low concentrations (Vrieze & Sorensen, 2001). For example, in the peacock blenny (*Salaria pavo*), females responded to water conditioned with reproductive males by swimming directly towards the source of the odour, yet females did not show any preference for water conditioned by heterospecific or non-reproductive males (Serrano *et. al.*, 2008). Similarly, reproductive female round gobies (*Neogobius melanostomus*) increased their swimming speed and spent more time

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near the odour source containing reproductive male conditioned water compared to control water (Gammon *et. al.*, 2005). Chemical signals induce releaser (behavioural) effects (e.g. Gammon *et. al.*, 2005; Serrano *et. al.*, 2008) and primer (developmental and/or endocrinological) effects (Sorensen & Stacey, 2004). Primer effects may not be observed for hours or days following the stimulus (Stacey, 1983). For example, in the common rudd (*Scardinius erythrophthalmus*) pre-ovulatory females induced changes in circulating hormone levels and milt volume of males so that milt stores would be increased by the time the female ovulated (Stacey *et. al.*, 2012).

Tactile reception is also expected to play a role in reproduction via vibrational courtship behaviours, especially in aquatic systems. For example, in the salamander (*Hynobius leechii*), males exhibit tail vibrations in all stages of courtship which are thought to help transport pheromones to females (Park *et. al.*, 1996). In fishes, tactile reception is carried out by the lateral line which is able to detect both moving and inanimate objects simply based on current-like water disturbances (Bleckmann, 1993), suggesting that females may be able to detect small disturbances generated by male fanning. For example, in the hime salmon (*Oncorhynchus nerka*), females will vibrate just prior to spawning for synchronization of gamete release (Satou *et. al.*, 1987). Similar vibrational behaviours have been observed in other fish, including the threespine stickleback (*Gasterosteus aculeatus*) (Wootton, 1976), and the grass goby (*Zosterisessor ophiocephalus*) (Marchesan *et. al*., 2000). In the grass goby, females perform fin oscillations creating small water vibrations signalling readiness to spawn (Marchesan *et. al.*, 2000). Fin oscillations (fanning) also are exhibited by nest-guarding male round

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gobies (Wantola, personal observation); however, the information which this behaviour conveys to potential mates is unknown.

The round goby, an invasive fish in the Laurentian Great Lakes and elsewhere (Corkum *et. al.*, 2004), occupies shelters with a single opening for nests (MacInnis and Corkum, 2000). The nests are found at depths of 2 to 11m and beyond where visibility is limited (Wickett & Corkum, 1998). Male round gobies are known to use pheromones (Gammon *et. al.*, 2005; Arbuckle *et. al.*, 2005) and possibly mechanosensory signals for mate attraction. Round gobies have specialized neuromasts (Charlebois *et. al.*, 1997), suggesting these fish may be more sensitive to movement than other fishes (Jude *et. al.*, 1995). During the spawning season, nest-holding males fan intermittently with their caudal fins pushing a plume of water out of the nest (Meunier, 2009; Chapter 2). Reproductive female round gobies often orient themselves perpendicular to nest-holding males so that the full length of their body is facing the nest (Meunier *et. al.*, 2009). Thus, reproductive females not only receive olfactory cues, but they also may monitor flow patterns created by male fanning. Both signals may be used in mate choice.

Gammon *et. al.* (2005) demonstrated a preference by reproductive female round gobies to the conditioned water of reproductive males. In this study, I further examined the difference in female responses to conditioned water of reproductive and juvenile males with the addition of changes in flow. I conducted a Y-maze (Grice box; Kolb *et. al.*, 1983) experiment in a laboratory flume where females (reproductive and non-reproductive tested separately) were given the opportunity to choose paired treatment combinations of conditioned water and flow. Because high quality males are thought to

pump water and release chemical signals out of nests, I predicted that reproductive

females would prefer higher flow conditions and conditioned water of reproductive males

even in the absence of males. I used an integrative approach by measuring behaviour,

GSI and reproductive hormones to elucidate patterns in female mate choice. The estrogen

(17β-estradiol) and GSI levels are important for reproduction and may help to clarify the

reproductive state of the female. For example, in the guppy (*Poecilia reticulata*) females

are more responsive to male courtship during parturition, which corresponds to

vitellogenesis of the next clutch of eggs and high 17β-estradiol levels (Liley,

1966;Venkatesh *et. al.*, 1990). Since round gobies have several clutches of eggs

developing simultaneously throughout the breeding season, I predicted that estradiol also

may play an important role in female receptivity of this species. I also predicted that

estradiol should be related to the GSI values such that estradiol should increase with

increasing GSI of non-reproductive females reaching a peak before decreasing prior to a

peak in GSI values that are exhibited by reproductive females (cf. Zeyl, 2012).

MATERIALS AND METHODS

Collection of Animals

Round gobies were collected by angling and seining on the Canadian shoreline of

the Detroit River (42°18'N, 83°04'W) and Lake Erie (42°15'N, 81°54'W), Ontario, April

to August 2012. The fish were brought to the Animal Care Facility at the University of

Windsor where they were tagged using visual implant elastomer (VIE) injectable tags

(Northwest Marine Technology) before the fish were placed into large (90 L) flow

through communal tanks. Each tank held about 20 fish. Reproductive females, which

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were separated from males to delay spawning, were placed into smaller (20 L) tanks. Fish

were kept at a constant 16L: 8D light regime and water temperature (20 ± 2° C) to simulate

conditions during the breeding season (Meunier *et. al.*, 2009). All tanks were lined with

aquarium gravel and contained several black PVC tubes and plastic square artificial nests

for shelter. Fish were fed daily with excess amounts of Nutrafin ® flakes and remaining

food was removed every 2-3 days as needed (Jones & Reynolds, 1999). All fish were

treated in accordance with the animal care protocols outlined by the Animal Care

Committee at the University of Windsor and the Canadian Council on Animal Care

Collection of Conditioned Water

Conditioned water (CW) was collected from reproductive (RM) and juvenile (JV)

male round gobies. Following the protocol of Gammon *et. al.* (2005), RM fish were

placed individually into glass jars containing 1 L of dechlorinated water along with an

aerator and left for 4 h. Each jar was covered with a black cloth to eliminate responses

induced by visual contact (disturbance) from other fish or surroundings. Similarly, JV

males were placed randomly in groups of 4 into glass jars to compensate for the

difference in mass between non-reproductive and reproductive fish. Males were

considered to be juvenile if they had small, unpronounced papilla and later (after

dissection) by the lack of gonadal development. After 4 h, fish were removed from the

water and returned to communal tanks. CW was passed through a vacuum filter and

Whatman (no. 1) filter paper to remove debris before combining water of either 5 RMs

or 8 JVs. Water of 5 RMs was pooled because not all males release steroids even though

they may show secondary sexual characteristics (Corkum & Cogliati, 2013). Filtered

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water was placed into 1 L aluminum water bottles in which the inside of the plastic lid was covered with aluminum foil. Water bottles were stored at -20°C until needed.

Behavioural Trials

I tested the preference of reproductive (RF) and non-reproductive (NRF) females, to flow rates (high flow, HF or low flow, LF) in the presence of CW from either reproductive (RM) or juvenile (JV) males. Six paired combinations of CW and flow (10 replicates each) were presented to either a RF or NRF: 1) reproductive male CW with high flow (RMH) vs. juvenile CW with high flow (JVH), 2) RMH vs. juvenile CW with low flow (JVL), 3) reproductive male CW with low flow (RML) vs. JVH, 4) RML vs. JVL, 5) RML vs. RMH, 6) JVL vs. JVH.

The experiment was conducted in a 1 m long flume, similar to that of Yavno & Corkum (2011). There was a 50 cm long, 30 cm high solid, opaque barrier, extending from the inflow end down the center of the flume to the mid-point of the flume (Figure 3.1). Two inflow valves (one for each arm of the maze created by the barrier) were at one end of the tank and a single, outflow valve was in the middle of the tank at the bottom. Inflow was set at either high (3.78 cm/s) or low (2.13 cm/s) flow on the basis of laboratory observations of males pumping water out of shelters via fanning (Chapter 2; Meunier, 2009). Small white particles that are neutrally buoyant in water were added to the tank and their movement was video recorded. A ruler was placed at the top of the tank so that the movement of particles could be recorded. When the male fanned using his caudal fin, particle movement was measured in cm/s to estimate flow patterns created by male fanning. Fanning was recorded for 5 males. The lowest and highest flow rates

observed were used for comparison in the flume experiment. Flow was controlled using two masterflex L/S variable-speed modular drives (ColeParmer; model: RK-7553-70). A 60 mL syringe was attached to each inflow using a 21 G 1½ gage needle so that, odours were gravity fed into the flume. After each trial, the flume, tubing and syringes were thoroughly cleaned and rinsed repeatedly.

Each trial consisted of an acclimation (1 h) and stimulus (15 min) period. The flow treatment, either high and/or low, was adjusted before a female round goby was added to the tank. At the start of the acclimation period, a female was selected at random and placed in the half of the tank away from the inflow. During the acclimation period, females were restricted to this area of the tank by a perforated divider which allowed continuous flow throughout the tank. During the last 30 s of the acclimation period, 125 mL of CW was added to the tank via the two 60 mL syringes (different syringes for RM and JV odours) attached to the inflow tubing. Treatment combinations were randomly assigned to the arms of the flume. The stimulus period began by remotely raising the divider, enabling the fish to explore both treatment arms. All trials were performed under dim light conditions as round gobies are more active at night (Dubs & Corkum, 1996). Trials were recorded using a video camera (Hitachi VKC-370) placed above the tank and connected to a DVD recorder (Sony RDR-GX330). Each fish was used only once.

Morphometric and Physiological Measurements

After each trial, the fish was sacrificed using clove oil (100 mg/L; Anderson _et. al._, 1997). Once a fish was dried with a paper towel to remove excess moisture, it was weighed to the nearest 0.01 g (Ohaus Adventure Pro AV412C digital balance). Total

length and Fulton's condition factor (K; weight/ length^3; Ricker, 1975) also were calculated for each fish.

Blood samples were collected from the caudal vein using heparinized micro-capillary tubes to measure blood plasma levels of 17β-estradiol (E2), a primary fish estrogen associated with the regulation of vitellogenin production in oocytes (Clelland & Peng, 2009). Plasma levels of E2 (ng/mL) were measured for individual fish to examine potential relationships between estrogen and behavioural responses in RF and NRF fish. In addition, E2 levels also were compared with time to first response and average swimming speed to determine if E2 levels influenced response behaviour. Each blood sample was transferred to a 1.5 mL Eppendorf tube and centrifuged at 13,000 RPM for 10 min to separate the blood plasma from the rest of the sample. The plasma was then separated and stored at -80°C until analyzed using a commercial enzyme linked immunosorbent assay (ELISA) for 17β-estradiol (Cayman Chemical, Ann Arbour, MI, USA; see Zeyl, 2012). Briefly, plasma for both RFs and NRFs fish was optimized to determine the optimal assay dilution and if extraction was required. All plasma was run un-extracted (i.e., 'raw') in triplicate at dilutions from 1:22.5-36 (based on plasma volume and reproductive status), and E2 ranged from 0.15 – 1.29 (ng/ml; n = 50) for RFs and 0.11 – 10.47 (ng/ml; n = 45) for NRFs. Intra- and inter-assay variability was 6.71% and 12.48%, respectively.

The gonadosomatic index (GSI; total gonad mass divided by the total mass x 100) was measured to determine the reproductive status of each female. Each female was dissected, gonads removed and weighed to the nearest 0.01 g before preservation in

ethanol. Females in this study were considered reproductive if their GSI > 8% and non-reproductive if GSI< 6% (Yavno & Corkum, 2011). Females with intermediate status (GSI between 6 and 8) were not used this study. The mean (standard error) GSI for RF and NRF was 11.67 ± 0.35 and 1.88 ± 0.22, respectively.

Data Analysis

Female choice of paired treatments for both RF and NRF was determined using a preference index (I) modified from Li *et al.* (2002). I = (TL/TT) – (TR/TT), where TL and TR were the total time spent in the left and right arms of the tank, respectively, and TT was the total time in the stimulus period (15 min). A series of 2-tail independent t-tests were used to determine differences in preferences between RF and NRF to each of the treatment combinations. All statistical analyses were performed in STATISTICA 7 (www.statsoft.com).

In order to compare behavioural responses with physiological measurements, I used a multiple correlation analysis (separate comparisons for RF and NRF). Behavioural responses that were used in the comparison included time to 1st response, average swimming speed. A cubic polynomial regression was used to describe the relationship between two physiological variables, estradiol and GSI.

RESULTS

Among all of the paired treatments, female choice differed significantly (df = 18, p = 0.05) in only one case, treatment 3 (RML vs. JVH). RF preferred RM CW and low flow conditions (2.13 cm/s), whereas NRF preferred JV CW and high flow conditions

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(3.78 cm/s). Overall, 66% of reproductive females and 71% of non-reproductive females initiated movement and explored the conditions in the arms of the flume.

Responses by RF and NRF differed significantly in their first response or "decision". RF chose the RML treatment first 48% of the time, whereas NRF chose RML first only 24% of the time (Figure 3.3). A more dramatic (7-fold) difference occurred between females in their first response to juvenile CW and high flow (JVH). NRF chose the JVH treatment first 34% of the time, whereas RF chose JVH first only 5% of the time. Both RF and NRF were similar in their first response decisions regarding the JVL (26% and 25% respectively) and RMH (21% and 17% respectively) treatments.

There was a significant relationship between the time to first response (time taken for fish to swim into one arm of the flume) and the average swimming speed for both RF and NRF. Overall, females that had a higher average swimming speed and made a first response decision faster than those fish with a lower average swimming speed regardless of reproductive status (n = 116, r = 0.75, p < 0.01). Those fish that swam faster also appeared to glide or coast between swimming bouts rather than pausing on the substrate. In addition, the average swimming speed of NRF also was related to condition factor (K). There was a significant positive relationship between K and average swimming speed of NRF (n = 40, r = 0.32, p = 0.043) (Figure 3.4a).

The GSI of RF was variable, ranging between 8.06 and 19.4. Average swimming speed and time to first response decisions differed with respect to GSI values of the RF. Response times varied inversely with GSI (n = 39, r = -0.32, p = 0.045), whereas there

was a significant positive relationship between average swimming speed and GSI (n = 43, r = 0.38, p = 0.011) (Figure 3.4b).

There was a significant quadratic relationship between E2 (Y) and GSI (X) of all females (Y = -0.017 + 0.28X – 0.33X^2 + 0.001X^3; p < 0.01). E2 levels increased with GSIs of non-reproductive females peaking at the transition between NRF and RF, after which E2 decreased with GSIs of reproductive females (Figure 3.5). There was no significant relationship between E2 and time to first response (RF n = 33, r = 0.24, p = 0.19; NRF n = 29, r = 0.24, p = 0.21) or average swimming speed (RF n = 32, r = -0.21, p = 0.25; NRF n = 29, r = -0.028, p = 0.89).

DISCUSSION

Female Responses to Conditioned Water and Flow

The olfactory system is well developed in fish and plays an important role in behavioural responses to stimuli, including both sexual and non-sexual stimuli (Liley, 1982) such as food (Constantino & Salmon, 2003), predators (Hazlett, 2007) or potential mates (Gammon *et. al.*, 2005). In the round goby, nest guarding males fan with their caudal fins which results in a plume of water being pushed out of the nest (Meunier, 2009; Chapter 2). It is likely that the constituents within the plume contain sex pheromones and so play a role in subsequent mate attraction (Arbuckle *et. al.*, 2005; Corkum *et. al.*, 2008). Females often orient themselves sideways towards males (Meunier *et. al.*, 2009), suggesting that in addition to receiving chemical stimuli via the olfactory system (Belanger *et. al.*, 2003), females may detect flow produced by fanning males.

There was a significant difference in the response of RF and NRF to treatment 3 (RML vs. JVH) whereby, RF preferred RML treatment and NRF preferred JVH treatment. Females also differed in their first response whereby RF chose RML most often and NRF chose JVH most often. The RF preference for reproductive rather than juvenile male CW is consistent with previous work by Gammon *et. al.* (2005), who showed that RF round gobies spent significantly more time near the CW of a RM than that of the control.

My findings also demonstrated that RF preferred low rather than high flow conditions. This preference for low flow was contrary to my prediction that increased flow created by male fanning should be a mechanism for mate attraction. There is an increased energetic cost associated with swimming against a current (Lester, 1971) and this cost is expected to be greater for gravid females because gravidity can impair swimming ability (Frommen *et. al.*, 2012). For example, in the Trinidadian guppy (*Poecilia* reticulata), swimming velocity and distance travelled declined as pregnancy progressed, suggesting a trade-off between reproductive costs and swimming performance (Ghalambor *et. al.*, 2004). Since females have already invested energy into egg production and because spawning increases metabolic stress (Wootton, 1974), RF may choose low flow conditions to conserve energy. I speculate that the preference for low flows exhibited by RF round gobies in my study may be explained by females lowering their energy costs.

Signal intermittency may be important for proper interpretation of signals because changes in the intermittency of a signal can elicit different responses (Kozlowski *et. al.*,

2003). For example, odour that was pulsed at specific rates enabled crayfish to successfully locate odour sources compared with continuous flow conditions (Kozlowski *et. al.*, 2003). In my experiment, females were exposed to continuous flow conditions, yet flow created by male fanning is often dispersed in short bursts. The lack of signal intermittency in my study may have accounted for the preference of low vs. high flow rates by RF.

In contrast to RF preference of RM CW dispersed at low flow, NRF preferred juvenile CW dispersed at high flow. The preference for the JVH treatment by NRF suggests that the CW from similar life stages may facilitate aggregation of the fish. Round goby preference to CW of RF by NRF, which was demonstrated by Gammon *et. al.* (2005), also suggests that pheromones may play a role in aggregation in the round goby. Also, Belanger *et. al.* (2006) showed that RF have strong EOG (electro-olfactogram) responses to CW of reproductive males while NRF do not. Corkum *et. al.* (2008) also demonstrated a trend for RF and NRF to prefer different blends of steroids (Conjugated vs. Free), illustrating the potential for pheromone based aggregation in the round goby. Average densities of the round goby in the western basin of Lake Erie range up to 1.8 individuals /m^2 (Johnson *et. al.*, 2005), but round goby densities up to 133 individuals /m^2 have been reported (Calumet Harbour, southern Lake Michigan) (Chotkowski & Marsden, 1999). This species clearly demonstrates social aggregation.

The trend for NRF to prefer high flow conditions could reflect the tendency for larger fish to occupy better (rocky) habitats while juveniles must occupy more open (sandy) habitats (Charlebois *et. al.*, 1997; Ray & Corkum, 2001; Johnson *et. al.*, 2005).

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Another possible explanation for NRF preference to high flow is that trials were conducted in the dark (night conditions). Since round gobies are thought move onto sandy beaches at night to feed (Jude *et. al.*, 1992; Dopazo *et. al.*, 2009), they may be attracted to conspecifics in high flow conditions in search of food.

Comparing Average Swimming Speed of Females

Females that had a faster average swimming speed also took less time to respond to treatments regardless of reproductive status. Round gobies are known to use two different swimming modes, "burst and hold" and "burst and coast". The "burst and coast" movement results in swimming speeds twice that of the "burst and hold" (Tierney *et. al.*, 2011). Both swimming movements were observed in my videos and those fish exhibiting the "burst and coast" appeared to swim faster because they paused less than those fish exhibiting the "burst and hold" technique.

An important note about female movement is that 34% of reproductive and 29% of non-reproductive females did not initiate movement and explore one of the treatment arms. These results are common in behavioural trials of the round goby (Corkum pers. comm.) however, the specific reason for this is unknown. These results do not appear to be related to time spent in the lab, GSI, condition of the fish or estradiol levels. Stress associated with handling time can result in changes to swimming and behavioural patterns (Barton, 2002) and may account number of fish that did not respond.

Average swimming speeds of NRF was positively related to condition factor. Condition is known to affect swimming ability in many different species. For example, condition was found to affect swimming capacity (Grorud-Colvert & Sponaugle, 2006),

critical swimming speed (Syriatowicz & Brooks, 2004), and swimming endurance

(Martinez *et. al.*, 2003), in the bluehead wrasse (*Thalassoma bifasciatum*), guppy

(*Poecilia reticulata*), and Atlantic cod (*Gadus morhua*), respectively. Condition can

affect the metabolic capacities of muscle by significantly reducing enzyme activity thus

affecting swimming performance (Martinez *et. al.*, 2003). These relationships suggest

that the overall condition of the fish may limit the amount of energy available and/or the

ability for faster swimming responses.

Average swimming speed and time to first response were positively related to the

GSI level of reproductive females. Costs associated with mate selection increase with

time (Bakker & Milinski, 1991) because ultimately gravid females are only able to hold

their eggs for a few days before they must either drop them or risk becoming egg bound

(Wooton, 1976), a stage known as the "latest spawning time" (Bakker & Milinski, 1991).

Reproductive female round gobies have three clutches of eggs during the reproductive

season (MacInnis & Corkum, 2000) and some eggs may be more hydrated (ripe) than

others. In my study, females with a higher GSI may have been close to their "latest

spawning time" because their eggs were more hydrated. The time limit placed on a

female's ability to spawn successfully would explain the rapid response time to stimuli of

reproductive male CW. Moreover, the RF that were not as close to their "latest spawning

time" may have had more time to evaluate the stimuli before responding.

Separation time between males and females likely affects mate choice. In the

threespine stickleback, females were shown to become less choosy when selecting a mate

as the interval between males increased (Milinski & Bakker, 1992). Luttbeg *et. al.* (2001)

also found that females were more likely to enter a male's nest when isolated from males for an increased period of time. Perhaps the gravid females in my study were increasingly time limited because of the holding time in isolated tanks and therefore were ultimately more receptive male stimuli.

Physiological Relationships Involving Estradiol

Estradiol (E2) is an important hormone in fish playing a vital role in gonadal development (Patiño & Sullivan, 2002). Plasma E2 levels were highly variable but were related to GSI levels of female round gobies, forming an inverted U-shaped relationship. Estradiol levels increased with the GSI of NRF with a peak around 8% before decreasing with GSI of RF. This relationship between E2 and GSI is consistent with the role of estradiol in vitellogenesis (yolk deposition). Estradiol stimulates the production of vitellogenin (Patiño & Sullivan, 2002) and peaks during vitellogenesis (Pankhurst *et. al.*, 1999) before declining during final maturation (Kime, 1993; Williams *et. al.*, 2004). Recently, Zeyl (2012) also showed that plasma E2 peaked around a GSI of 8% in the round goby. A similar relationship was observed in the swordtail (*Xiphophorus nigrensis*) where females undergoing vitellogenesis had a higher plasma E2 level than those females that were in the final stages of egg development (Ramsey *et. al.*, 2011). Sisneros *et. al.* (2004) also noted that in the plainfin midshipman (*Porichthys notatus*) E2 levels are highly variable especially during the pre-nesting stage.

Plasma E2 levels were not significantly related to any behavioural displays that were examined (i.e. time to first response, average swimming speed etc.) of female round gobies. Work on the spiny damselfish (*Acanthochromis polyacanthus*) demonstrated that

plasma E2 levels were unrelated to behaviour within a gonadal stage and therefore, may not influence reproductive behaviour (Pankhurst *et. al.*, 1999). Similarly, in the swordtail, females with higher E2 levels were less likely to display preference behaviours towards males (Ramsey *et. al.*, 2011). Ramsey *et. al.* (2011) also noted that estradiol was a significant negative predictor of preference measures but was positively related to locomotion. Overall, these studies suggest that E2 may be unable to elicit sexual behaviour in female fishes (Munakata & Kobayashi, 2010).

In summary, reproductive and non-reproductive female round responded to opposite flow and conditioned water stimuli. RF preferred the CW of RM and the low flow conditions, whereas NRF preferred CW of juvenile males and high flows. Evidently, energetic costs of egg production and female condition may be important in mate choice (Wootton, 1974; 1976). The attraction of NRF to the CW of other juveniles suggests that chemical signals may play a role in aggregation in young gobies. This aggregation behaviour accounts for the high densities of round gobies reported throughout the Great Lakes region (Chotkoski & Marsden, 1999; Johnson *et. al.*, 2005). Overall, the differential responses by reproductive and non-reproductive females to CW and flow help to explain mate attraction and aggregation in the round goby. Further research into the relative importance of signal intermittency by nesting males and assessment by females may clarify mate choice in this species.

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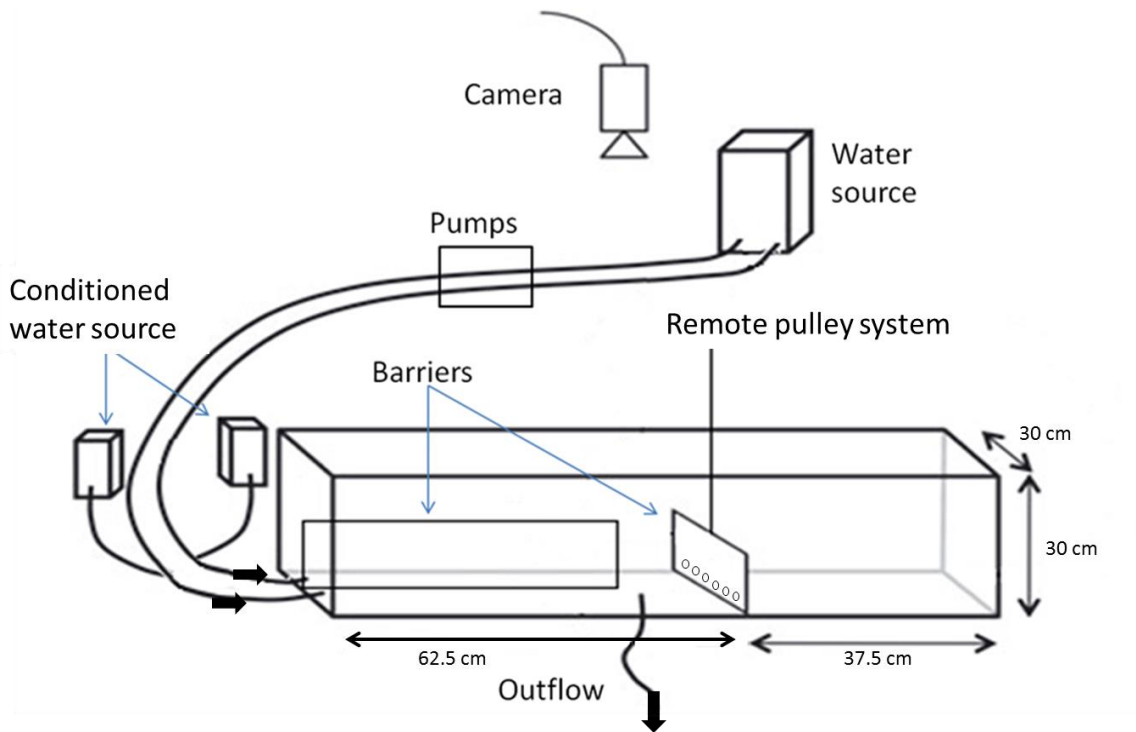
Figure 3.1 Illustration of Y-maze flume used to test female preference. Females were kept isolated on right side of the tank during the 1 hour acclimation period before the middle barrier was raised using the remote pulley system to allow females to explore the trial arms (see methods).
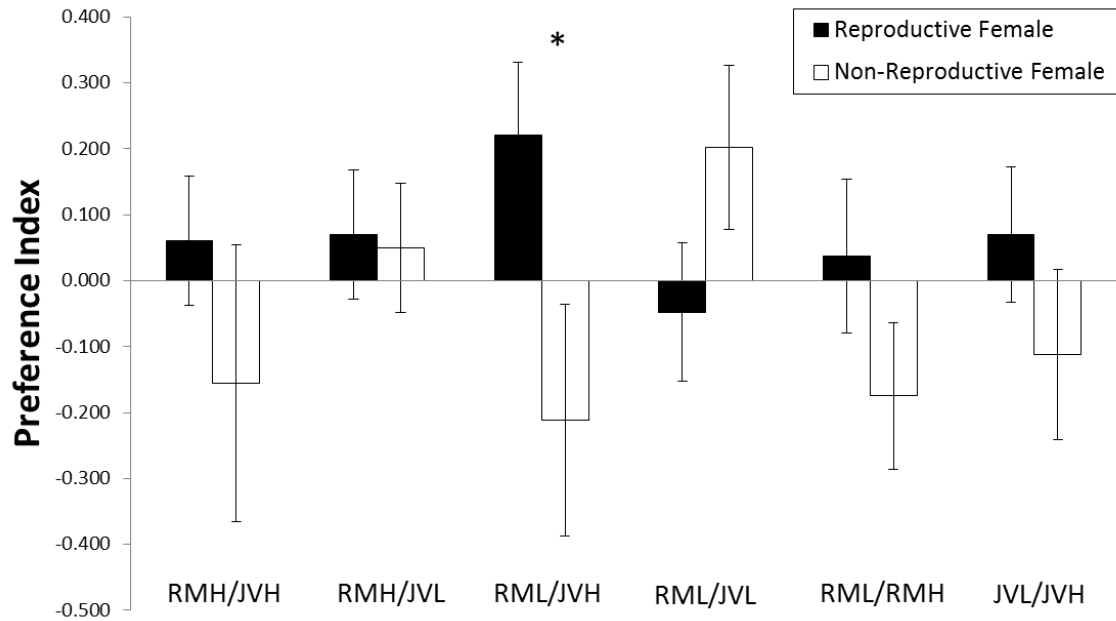
Figure 3.2 Preference index for time spent in each arm of the flume by reproductive (black bars) and non-reproductive (white bars) females in response to 6 paired treatments. A positive value represents mean (SE) time spent in the arm of the 1st treatment of the combination listed below the graph; a negative value represents mean (SE) time spent in the arm of the 2nd treatment of the combination listed below the graph. A sample size of 10 was used for each of the treatments with the exception of RML/RMH (n = 9) and JVL/JVH (n = 10 and 8 respectively). Codes are: RM, reproductive male odour; JV, juvenile male odour; H, high flow; L, low flow.

Reproductive Females

Non-Reproductive Females
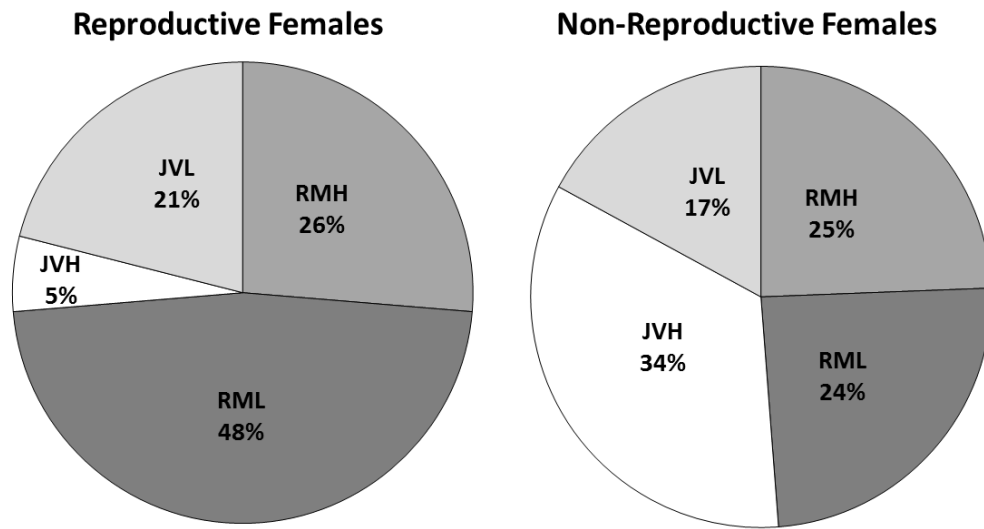
Figure 3.3 The pie charts represent the first treatment selected by reproductive and non-reproductive fish during the stimulus period. Codes are: RM, reproductive male odour; JV, juvenile male odour; H, high flow; L, low flow.
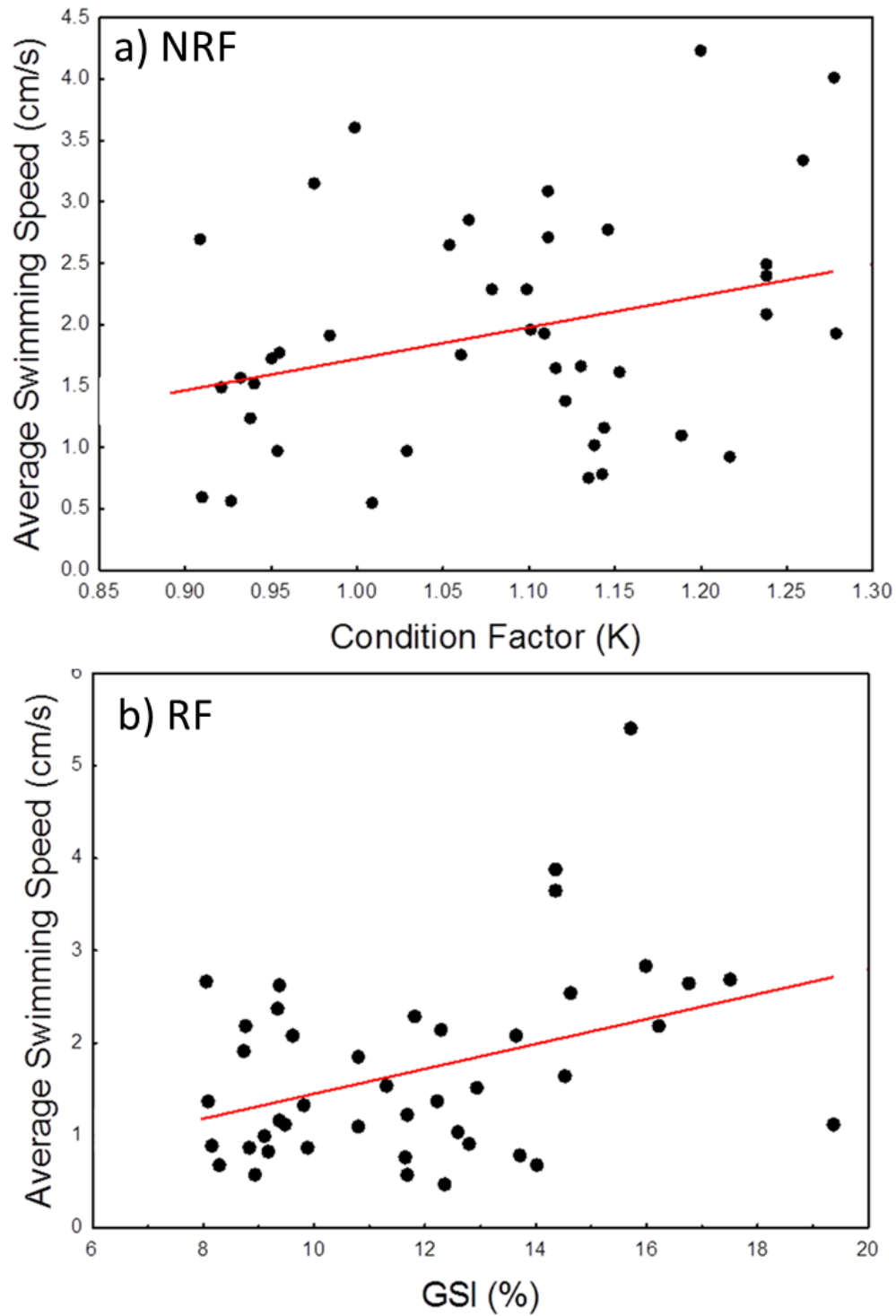
Figure 3.4 The relationship between average swimming speed and a) condition factor of non-reproductive females (n = 40, r = 0.32, p = 0.043) and b) GSI of reproductive females (n = 43, r = 0.38, p = 0.011).
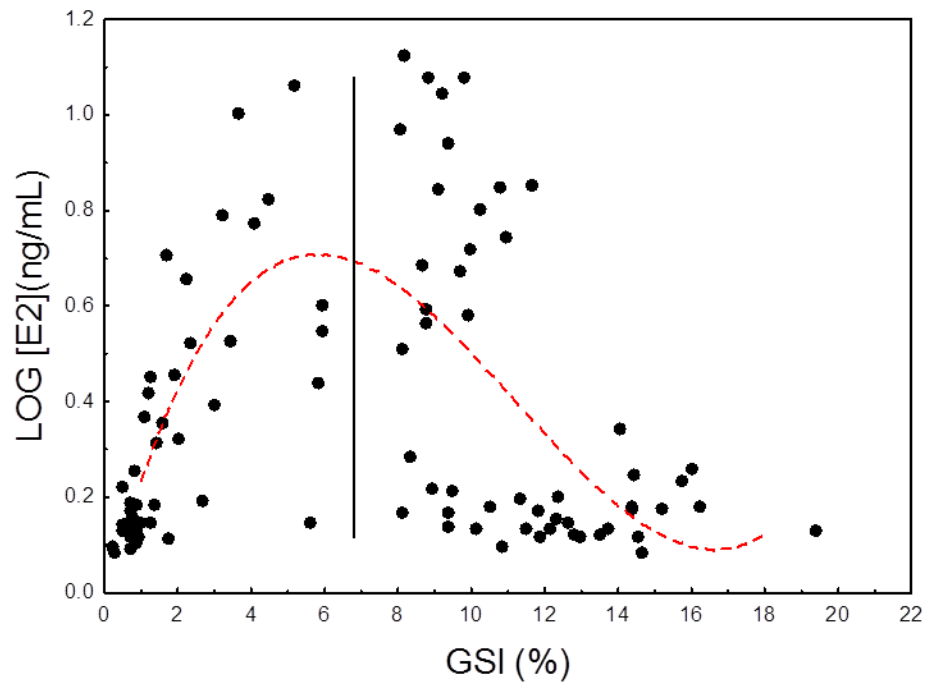
Figure 3.5 The relationship between plasma E2 levels and GSI for reproductive (right of the solid line, GSI > 8%) and non-reproductive (left of the solid line, GSI < 6%) females. The bold dotted line represents the significant quadratic relationship between E2 and GSI (see results).

CHAPTER 4 – GENERAL DISCUSSION

Sensory communication is important for reproduction in many fishes, including visual (Andersson, 1994), olfactory (Wyatt, 2003) and mechanosensory signals (Bleckmann, 1993; Sargent *et. al.*, 1998). Elaborate sexual ornaments (i.e. fin elongations) although potentially costly can provide important information to potential mates about an individual's quality (Bakker & Mundwiler, 1999). Visual signals however, can be limited by environmental conditions such as turbidity, increasing the importance of olfactory and mechanosensory signals under such condition. Sensory signals are not mutually exclusive and many fishes may rely on multiple signals for communication (Candolin, 2003). For example, in the swordtail (*Xiphophorus pygmaeus*) females use both visual and chemical signals in mate choice to avoid mating with male heterospecifics that can be attractive because of their larger size, one of the traits preferred by females (Hankison & Morris, 2002).

The round goby (*Neogobius melanostomus*) is an invasive species of fish in the Great Lakes region whose aggressive nature and high fecundity have allowed it to out-compete many native species for food and nest sites (Dubs & Corkum, 1996; MacInnis & Corkum, 2000). The specifics of round goby reproduction were recently characterized by Meunier *et. al.* (2009). Round goby males will guard a nest during the reproductive season and attract several females to the nest to spawn (Meunier *et. al.*, 2009). Males also have been shown to use pheromones as a method of mate attraction (Gammon *et. al.*, 2005; Arbuckle *et. al.*, 2005). However, there is a lack of information, detailing the specifics of communication between male and female round gobies during the pre-

spawning period. In addition, it is difficult to determine reproductive status of male round gobies as two males with the same gonadosomatic index (GSI) value may have different androgen levels (Corkum & Cogliati, 2013). The exact method by which female round gobies assess potential mates remains unknown.

Parental care is common among species that exhibit care with 50% exhibiting male only care (Gross & Shine, 1981; Sargent, 1997). Male parental care is exhibited in the round goby, including fanning of developing embryos using pectoral fins (Wootton, 1976; Sargent, 1997; Meunier *et. al*., 2009). This fanning behaviour is important as it provides oxygen to developing embryos, which is required for egg survival (Lindström & Hellström, 1993; Östlund & Ahnesjö, 1998; Meunier *et. al*., 2009). In addition to this type of fanning, males exhibit both pectoral and caudal fin fanning behaviours prior to egg deposition (displacement fanning). However, few studies have examined the role of displacement fanning in reproduction. Meunier *et. al*. (2009) speculated that displacement fanning may play a role in pheromone dispersal allowing a male to push water farther and faster out of the nest. Other researchers have suggested that, fanning may play a role in female mate choice as an honest indicator of male parental abilities (Sevenster, 1961; Bakker & Mundwiler, 1999). Despite these suggestions, there is little information on whether females may be able to directly detect changes in flow created by male fanning or whether these changes in flow may play a role in mate choice.

The goal of my research was two-fold. My first objective was to determine if displacement fanning was related to male condition or reproductive state. My second objective was to determine how females respond to changes in flow (typical of high and

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low male fanning rates) in the presence of conditioned water from reproductive and juvenile male round gobies.

Based on my findings, I conclude that not only fanning but the size of the fins themselves may be important courtship displays by the male round goby. I support this idea in Chapter 2 whereby reproductive male round gobies were found to have proportionally larger fused pelvic and caudal fins than non-reproductive males. I propose that a larger caudal fin may be important during lifting behaviours to make the male appear larger. This illusion of a larger appearance could benefit the male directly as females often prefer to mate with larger males (Lindström, 1992). Also, larger males often win in male-male competitions, allowing them to be better able to defend the nest against potential predators (Magnhagen & Kvarnemo, 1989). I support the notion that fanning may aid in the dispersal of pheromones (Meunier *et. al.*, 2009). Specifically, large caudal fins enable males to efficiently push water (and potentially, sex pheromones) out of the nest (Bakker & Mundwiler, 1999). Since females were attracted to low flow conditions (Chapter 3), larger caudal fins may allow a male to efficiently create small currents without a large energy expenditure. Energy may be more effectively allocated for parental care and mate attraction. I also suggest that fin size (surface area) may be a potentially useful less invasive measure of reproductive status in the round goby.

In Chapter 2, I also found those males that fanned more frequently with their pectoral fins also fanned for longer periods of time. I propose that this may be a reproductive strategy used by males to maximize the number of spawning events in a single season because pectoral fanning could be indicative of parental abilities. This also

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was demonstrated in the threespine stickleback (*Gasterosteus aculeatus*) as males that fanned more frequently and for longer periods of time were able to get females to spawn more easily (von Hippel, 2000). Increases in pectoral fanning have been associated with increases in weight loss and corresponding decreases in condition of nest-guarding male sand gobies (*Pomatoschistus minutus*) (Jarvi-Laturi *et. al.*, 2008). Males must increase their fanning as eggs develop to meet oxygen demands of the developing embryo or risk an unsuccessful brood (Lindström & Wennström, 1994), suggesting a trade-off between mate attraction and hatching success.

Males increase fanning behaviours in the presence of females, which suggests that males may relay information about their condition to females through fanning. Interestingly, I noted a negative relationship between maximum pectoral fanning rate and male condition (Chapter 2) which supports the idea suggested by Rohwer (1987) that males of lower condition may try to deceive females about the presence of eggs in nests by excessive fanning in order to gain additional spawning events. This idea is consistent with several hypotheses that explain why females may prefer to spawn with males whose nests already contain eggs, including female copying (Jamieson, 1995) and reductions in filial cannibalism (Rohwer, 1978; Forsgren *et al.*, 1996). In addition, I observed a positive relationship between plasma 11-ketotestosterone (11-KT), a primary fish androgen associated with secondary sexual characteristics (Borg, 1994), and maximum pectoral fanning rate (Chapter 2). Male bluegill sunfish that were able to nest multiple times in a single season had higher 11-KT levels and were in better condition than those

males that nested only once (Magee *et. al.*, 2006). Therefore, I suggest that males may be signalling condition and androgen levels through fanning behaviours.

Olfaction in fish is highly developed and pheromones play a role in mediating sexual behaviours such as mate attraction because pheromones are able to travel long distances (Liley, 1982). Moreover, pheromones are detectable at low concentrations (Vrieze & Sorensen, 2001). In addition to olfaction, tactile reception, which is mediated by the lateral line, also plays a role in mediation reproduction however this is less well studied. In Chapter 3, I found a preference by reproductive females to low flow conditions and the conditioned water of reproductive males. Previous work by Gammon *et. al.* (2005), also demonstrated a preference by reproductive female round gobies to conditioned water of reproductive males as females spend significantly more time near the male stimulus than that of the control. The preference by reproductive females to low flow conditions was contrary to our prediction that increases in flow created by male fanning should be a mechanisms for mate attraction. The low flow preference by reproductive females may be explained by a trade-off between reproductive costs and swimming performance (Ghalambor *et. al.*, 2004) because there is an increased energetic cost associated with swimming against a current (Lester, 1971) and this cost is greater for reproductive females (Wootton, 1976).

Signal intermittency may have also played an important role in the observed preferences by females to conditioned water and flow because changes in intermittency of a signal can elicit different behavioural responses (Kozlowski *et. al.*, 2003). In Chapter 3, females were exposed to continuous flow conditions within the experimental maze, yet

flow created by male fanning is often dispersed in bursts of varying lengths (Östlund &

Ahnesjö, 1998). Given that, reproductive males that fanned at a lower rate also fanned for

a longer period of time (Chapter 2), we would expect that continuous flow conditions

simulate these males rather than males that fanned in shorter bursts. This may explain

female preference for low flow conditions. As stated previously, fanning requires a larger

energy expenditure and higher pectoral fanning rates are associated with increases in

weight loss (Jarvi-Laturi *et. al*., 2008). I propose that this increased energy expenditure

would also apply to caudal fanning rates whereby males with lower caudal fanning rates

may expend less energy than those males with higher caudal fanning rates. Given that a

lower fanning rate may result in a lower energy expenditure, females may prefer to mate

with males creating a lower flow (lower fanning rate) because these males may have

higher energy reserves. This increase in energy reserves may allow the male to provide

higher quality parental care (Unger & Sargent, 1988) and exhibit a higher reproductive

success (von Hippel, 2000; Ito *et. al*., 2010).

Reproductive females are under considerable time constraints during the

reproductive season and the costs of mate selection increase with time (Bakker &

Milinski, 1991). Gravid females are only able to hold their ripe eggs for a few days

before they must decide to either drop them or risk becoming egg bound (Wootton,

1976). The point at which the fish must make the decision to drop or resorb eggs is

known as the "latest spawning time" (Bakker & Milinski, 1991). In Chapter 3,

reproductive female round gobies with higher GSI levels swam faster and responded

more quickly to stimuli. I propose that females with increased GSI levels were closer to

their "latest spawning time" and therefore responded more rapidly to reproductive male stimuli because there was only a small window of opportunity in which the female could successfully spawn.

Summary and Future Directions

Through analyses of male features and observations of male pre-spawning behaviour I have illustrated the importance of fin size and displacement fanning in the reproductive behaviour of the round goby. I also investigated how fanning behaviour may relate to male reproductive state or condition although the specific costs associated with fanning in the round goby are still unknown. Additional research into different types of costs associated with male fanning (i.e. energetic, increased visibility to predation) may further explain behavioural patterns and relationships observed in this study. In addition, examining what costs are associated with fanning may provide further insight into which aspects of fanning are most costly. This information may illustrate which features of fanning behaviour (rate, frequency, duration etc.) may be used by females to measure quality if different aspects of fanning behaviour are not equally costly to display. Courtship behaviours of male and female threespine sticklebacks (*Gasterosteus aculeatus*) changed in the presence of a predator (Candolin, 1997). It would be interesting to determine if male round gobies also change their fanning pattern in the presence of intruders and how females may respond to this change in fanning behaviour. Alternatively, females may prefer males that continue to display courtship behaviours under less ideal conditions. For example, in the amur goby (*Rhinogobius brunneus*) females prefer males that court in faster water currents because this is an honest indicator

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of parental quality (Takahashi & Kohda, 2001; Takahashi & Kohda, 2004). Also, I have proposed an additional non-invasive measure of reproductive status that in conjunction with current methods may aid in the clarification of reproductive status. Findings of my study highlight the importance of using multiple methods in the determination of reproductive status because the inherent variability of a single metric may be misleading.

Using laboratory experiments, I determined female preference for conditioned water of reproductive males and low flow conditions, suggesting that females may use currents created by male fanning to evaluate potential mates. Displacement fanning may serve as courtship behaviour and provide information to the female about male condition or parental abilities. Further research that investigates fanning and mate choice throughout the brooding cycle in this species may illustrate which information is most important to females when selecting a mate. Many females prefer to mate with males that already contain eggs in their nest and several hypotheses have been suggested for this, including mate copying and decreased risk of egg cannibalism (Forsgren *et. al.*, 1996). It would be interesting to determine if female round gobies show this same preference and whether preference behaviours can be attributed to one or more of these hypotheses.

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APPENDICES

APPENDIX A

ELISA Protocol for 11-ketotestosterone

Buffers and Solutions

Coating buffer
      1.59 g Na_2CO_3
      2.93 g $NaHCO_3$
Dissolve above chemicals in 1 l of DDW (double distilled water) and adjust pH to 9.6 by adding about 10 ml HCl (1 mol/l) then filter through Sep-Pak® C18.

Assay Buffer
      2.42 g Trishydroxyaminomethane (20 mmol/l)
      17.9 g NaCl (0.3 mol/l)
      1 g Bovine serum albumin (added after pH has been adjusted)
      1 ml Tween 80
Dissolve above into 1 l DDW and adjust pH to 7.5 by adding about 10 ml HCL (1 mol/l) then filter through Sep-Pak® C18

2nd Coating Buffer
      3.146 g Trishydroxyaminomethane (20 mmol/l)
      23.3 g NaCl
      13 g BSA
      1.3 g Sodium azide
Dissolve above into 1.3 l of DDW and adjust pH to 7.5 with about 40 ml HCl (1 mol/l) and filter through Sep-Pak® C18

Washing Solution
      0.5 ml Tween 20
      2.5 l DDW

Substrate Buffer for Peroxidase
      1.36 g Sodium acetate (10 mmol/l)
Dissolve into 1 l DDW and adjust pH to 5.0 with approximately 8 ml of citric acid (5%)

Enzyme Solution for Streptavidin-reaction
      30 ml assay buffer
      1 ul streptavidin-POD-conjugate (0.5 U)
Mix on a magnetic stirrer for a few minutes

Substrate Solution for Peroxidase
>   30 ml of substrate buffer
>   500 ul 3,3',5,5'-tetramethylbenzedine (0.4%)
>   100 ul H_2O_2 (0.6%)
Mix above gently on magnetic stirrer for a few minutes

Stop Reagent
>   900 ml DDW
>   100 ml H_2SO_4 (95-97%)
Mix on a magnetic stirrer

Standards
1. Take 10 ul of stock solution (1 mg/ml?) and add it to 990 ul of assay buffer (10 ng/ul)
2. Take 10 ul of previous solution and add it to 990 ul of assay buffer (100 pg/ul)
3. Take 100 ul of previous solution and add to 213 ul of assay buffer (32 pg/ul)
4. Add 25 ul of previous solution to given well of plate
   ➢ Dilutions in plate will become 400 pg/well then 100 pg/well and 25 pg/well etc.

Enzyme label
1. Take 10 ul aliquot and add to 1 ml of assay buffer (=1:1000)
2. Take 12.5 ul of previous solution and add to 12.5 ml assay buffer (this solution can be frozen for use over a few days; =1:100K) in test tube
3. Place parafilm on test tube and shake gently for a few seconds before use

Antibody
1. Take 25 ul of 1:100 antibody from the fridge and add to 12.5 ml of assay buffer in test tube
2. Place parafilm on test tube and shake gently for a few seconds before use

Plate Coating

Day 1
1. For one plate make a solution of 50ug protein A dissolved in 25ml of coating buffer
2. Put 0.25ml/well of the previous solution in each well then cover and let sit at room temperature overnight

Day 2
1. Discard solution from previous step and fill each well with 0.3ml of the second coating buffer

2. Cover with dust cover and parafilm and keep it at room temperature until needed (cand be used after 3 hours and do not keep at room temperature for more than 4 weeks however they can be frozen at -20°C for longer)

Assay Protocol

Day 1
1. Preload non-sticky plate with 75 ul of assay buffer
2. Load 25 ul of standard into well 1 of row G and H
   ➢ Transfer 45ul from well 1 into well 2 then from 2 into 3 etc. (mix slightly with pipette each time).

Full colour = no sample or standard (this is blank)
Zero = no antibody
* top off missing volume with assay buffer*

3. Add sample to well 1 of appropriate row and transfer in same method as for standard
4. Wash sticky plate 2x in machine with wash buffer
5. Transfer 50 ul of each well on non-sticky plate into corresponding well on sticky plate
   make sure to change pipette tips
   ➢ Wash out non-sticky plate with water when done
6. Add 100 ul of enzyme label to sticky plate
   note wells without enzyme label, remove pipette tip for those wells
7. Add 100 ul of antibody to sticky plate
   note wells without enzyme label, remove pipette tip for those wells
8. Place sticker on plate and seal tightly then cover with a damp paper towel and place in plastic bag.
9. Shake lightly for a few minutes and refrigerate overnight

Day 2

10. Wash plate 2x in machine with wash buffer
11. Add 250 ul streptavidin solution to each well
    must use larger blue pipette tips
12. Cover plate with sticker and damp paper towel then put in plastic bag and shake gently for 45 minutes
13. Wash plate 2x in machine with wash buffer
14. Add 250 ul of substrate solution to each well
    must use larger blue pipette tips
15. Cover plate with sticker (but no paper towel) and place in plastic bag and shake for ~45min (can be up to 2h) or until you begin to get a gradient with a slight blue tinge to the highest concentration well (left side)

16. Add 50 ul of sulfuric acid solution to each well

Place sticker on plate and put in plastic bag then shake for a few minutes before analysis with computer. Analysis done at 450 nm (0.1s)

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