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Condition-dependent auditory function and reproductive development in the round goby, *Neogobius melanostomus*

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Condition-dependent auditory function and reproductive development in the round goby,
Neogobius melanostomus

by

Jeffrey N. Zeyl

A Thesis
Submitted to the Faculty of Graduate Studies
through Biological Sciences
in Partial Fulfillment of the Requirements for
the Degree of Master of Science at the
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Windsor, Ontario, Canada

2012

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{May, 15, 2012}

DECLARATION OF ORIGINALITY

I. Co-Authorship Declaration

I hereby declare that this thesis incorporates material that is result of joint research, as follows:

This thesis also incorporates the outcome of a joint research undertaken in collaboration with Dr. Oliver Love and Dr. Dennis Higgs. The collaboration is covered in Chapter 2 of the thesis. In all cases, primary contributions, experimental designs, data analysis and interpretation, were performed by the author, and the contribution of co-authors was primarily through the provision of advice on experimental design, interpretation and analysis.

I am aware of the University of Windsor Senate Policy on Authorship and I certify that I have properly acknowledged the contribution of other researchers to my thesis, and have obtained written permission from each of the co-author(s) to include the above material(s) in my thesis.

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II. Declaration of Previous Publication

This thesis includes [1] original papers that have been previously published/submitted for publication in peer reviewed journals, as follows:

Thesis Chapter	Publication title/full citation	Publication status*
Chapter 2	Condition-dependent auditory function in the round goby, <i>Neogobius melanostomus</i>	In prep. for Journal of Experimental Biology

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ABSTRACT

Neural responses to sensory stimuli often differ between sexes and can be regulated by endocrine activity. This thesis examines the effects of sex, reproductive condition, female plasma 17β -estradiol level, and saccule hair cell density on auditory function in the round goby (*Neogobius melanostomus*). Relative to males, females had greater auditory sensitivity in the upper range of their hearing (300-600 Hz) and a higher density of hair cells. Female 17β -estradiol was associated with changes in auditory filtering properties at low frequencies (100-200 Hz). Additionally, I examined associations between gonadosomatic index, reproductive hormones, and stage of gonadal development in the round goby. Gonadosomatic indices provided limited resolution on reproductive condition in males and females; these categories encompassed individuals in varied endocrine and gonadal conditions. The results demonstrate auditory sexual dimorphism, elucidate the physiological mechanisms regulating auditory function, and present a framework for future studies on the reproductive cycle in the round goby.

DEDICATION

To Mom and Dad for all their love and support.

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CHAPTER I: INTRODUCTION

Introduction Part A

Role of sensory systems in signal evolution and sexual selection

In animal communication, signal characteristics and receiver sensory traits are expected to coevolve (Endler, 1992). In the context of courtship signalling, the evolution of male signals should be limited by the properties of the receiver's sensory system; similarly, sensory systems that are effective in receiving male signals should be favoured (Guilford and Dawkins, 1991, Endler, 1992, Ryan 1998). When acoustic communication is favoured by natural selection, "matched filters" - where sensory traits match characteristics of the signal - are predicted to evolve (Endler, 1992, Henry and Lucas, 2008). However, sensory systems also evolve outside of the communication contexts (e.g. predator avoidance, foraging), resulting in a mismatch between signaller and receiver properties (Ryan et al., 1990, Bailey and Romer, 1991, Kostarakos et al., 2008). Thus, it is critical to examine sensory function to understand the limits on the evolution of communication systems.

Acoustic communication is a central feature of reproductive behaviour in many taxa (Wells 1977, Bass and McKibben, 2003). The auditory system plays a central role in reproductive outcomes because it determines how efficiently acoustic signals are detected and encoded. Chapter 2 of this thesis focuses on how two aspects of receiver condition - sex and reproductive state - may influence auditory function within a species.

Condition dependent responses in receivers

Receiver sex

Sex differences in auditory sensitivity could also evolve when males and females differ in the effective range used for auditory reception (Gall et al., 2011). This scenario could occur in territorial species where females arrive briefly on the breeding grounds to mate with males, relying heavily on acoustic cues for navigation. An example of this occurs in spring peepers (*Hyla crucifer*), in which females have lower auditory thresholds than males at frequencies corresponding to male advertisement vocalizations (Wilczynski et al., 1984). This sensitivity difference gives females a signal detection range that is approximately 6 times greater than males (Brenowitz et al., 1984).

Interestingly, males position themselves such that the sound levels of acoustic signals are near auditory threshold as it reaches neighbouring males (Brenowitz et al., 1984). This sex difference in hearing ability apparently facilitates male spacing, while still maintaining sufficient cohesion to attract females to the breeding grounds (Brenowitz et al., 1984). Sex differences in auditory function have been observed in various taxa, including humans (McFadden, 1998), birds (Gall et al., 2011), frogs (Wilczynski et al., 1984, Miranda and Wilczynski, 2009), and insects (Bailey and Romer, 1991).

Males and females often both respond to the same sexual signals (Berglund et al., 1996), but the optimal responses may differ between sexes (e.g. phonotaxis and mate choice in females vs. agonistic displays in males). Sex differences in auditory function may evolve when the sexes differ in the relative costs of misidentification of heterospecific signals (Searcy and Brenowitz, 1988). Female attraction to heterospecific courtship signals (an acoustic identification error) should come at a high cost due to the

wasted hybrid mating (Searcy and Brenowitz, 1988, Bernal et al., 2009). Females that are more selective in responding to acoustic signals should experience higher reproductive success than indiscriminate females. In contrast, the cost of misidentification could be lower in males; it is the cost associated with displaying to another male when there is no threat. Thus, in some mating systems, females may be more selective than males in their discrimination of acoustic signals, which is supported by behavioural studies (Searcy and Brenowitz, 1988, Bernal et al., 2007). Greater auditory selectivity in females could result from sex differences in auditory filtering properties in the auditory periphery or midbrain.

Receiver reproductive state

Acoustic signal output often increases during the breeding period in vertebrates (Wells, 1977, Tramontin et al., 2000, Kasumyan, 2009), and behavioural responses to acoustic signals similarly vary seasonally and/or with reproductive status. For example, in the tungara frog, *Physalaemus pustulosus*, the probability of female approach toward male vocalizations is greater when in an amplexed stage (Lynch et al., 2005). Similar reproductive state-dependent phonotaxis results have been found in the plainfin midshipman fish, *Porichthys notatus*, where gravid females exhibit a greater probability of phonotactic responses to acoustic signals than spent females (Bass and McKibben, 2003).

Seasonal and reproductive state-dependent responses to acoustic signals could be regulated by gonadal hormones. Gonadal hormones are thought to be effective mechanisms for permitting or triggering mating behaviour in 'associated' mating

systems, where mating behaviours occur in close temporal association with gonadal hormone elevation and gonad development (Crews, 1984, Crews and Moore, 1986, Nelson et al., 1990). Gonadal hormones have been found to influence reproductive behaviours across taxa (Remage-Healey and Bass, 2006, Munakata and Kobayashi, 2010, Lynch and Wilczyński, 2011), and to modulate phonotaxis preferences (Lynch et al., 2006, Gordon and Gerhardt 2009). Moreover, hormone elevations often occur at a time when animals are performing phonotaxis and other acoustic discrimination tasks (Sisneros et al., 2004, Gordon and Gerhardt, 2009). Thus, behavioural responses to acoustic signals hormones may be under endocrine control.

Hormones could affect responses to acoustic signals by altering how acoustic signals are received and processed in the auditory system (i.e. changing in sensitivity or coding properties). There are a growing number of studies indicating that auditory function varies seasonally and/or with endocrine state (Sisneros et al., 2004, Goense and Feng, 2005, Bass and Zakon, 2005, Caras et al., 2010). Several songbirds have higher auditory evoked potential (AEP) amplitudes in the spring relative to the fall, which parallels seasonal changes in vocal activity (Lucas et al., 2002, 2007). Testosterone implants increase midbrain auditory threshold in green treefrogs, *Hyla cinerea* (Miranda and Wilczyński, 2009), and male northern leopard frogs, *Rana pipiens*, experience a shift in auditory frequency tuning in the late spring and summer, relative to winter (Goense and Feng, 2005). In fish, gonadal steroids affect electroreception and audition tuning (Sisneros and Bass, 2003, Bass and Zakon, 2005). Auditory function in human females varies across the menstrual cycle (Elkind-Hirsch et al., 1992, Walpurger et al., 2004). There is also evidence of direct hormonal on the peripheral auditory system; androgen

and/or estrogen receptors have been found in the peripheral auditory nerve afferents and/or inner ear in birds (Noirot et al. 2009), mammals (Stenberg et al., 1999), and fish (Forlano et al., 2005, 2010, Maruska and Fernald, 2010).

Acoustic communication in fishes

Many species of marine and freshwater fish use acoustic communication during behaviours related to territoriality, aggression, courtship, and mating (Zelick et al., 1999). In fish occupying shallow water environments (e.g. intertidal zones, lake and river shorelines), acoustic communication may be limited by physical properties of sound propagation. The ability for sound to propagate in shallow water depends on water depth and substrate type, among other factors (reviewed by Bass and Clark, 2003). As depth decreases, the cutoff frequency above which sounds will propagate also increases (Bass and Clark, 2003). Similarly, as the speed of sound in the substrate decreases, the cutoff frequency also increases (Bass and Clark, 2003).

Additionally, most vocalizing species do not have auditory specializations to detect pressure waves, limiting hearing to detection of local water motions (Popper and Fay, 2011). The absence of pressure detection can limit the frequency range of hearing to below 500 Hz in many species (Ladich and Bass, 2003). Thus, in contrast to much acoustic communication in the open ocean and on land, where sound may be used over large ranges (Forrest, 1993), acoustic communication in shallow water fishes is typically used for short range signalling (< 10 m, Bass and Clark, 2003).

The most common context for sound production in fishes is during the spawning season (Zelick et al., 1999, Kasumyan, 2009). Vocalizations are commonly produced by

males defending territories and serve to attract females or to mediate agonistic interactions with other males (Ladich 1997, Kasumyan, 2009). Fish sounds are typically a collection of simple pulse sounds of short duration (<1 s) with little frequency modulation (Bass and McKibben, 2003, Ladich and Bass, 2003). Most of the differences between closely related species occur in temporal parameters such as pulse duration and inter-pulse interval (Ladich 1997, McKibben and Bass, 2003). Vocal production can influence mate choice (Myrberg et al., 1986, Verzijden et al., 2010) and determine reproductive success (Vasconcelos et al., 2012).

Study species: Round goby

In Chapter 2, I examined effects of sex, reproductive condition, and endocrine state on auditory reception in a vocal teleost fish, the round goby, *Neogobius melanostomus*. Like other freshwater gobies (Lugli et al., 1997; Bass and McKibben, 2003), male round gobies produce pulse sounds during reproductive periods, presumably for mate attraction and/or to ward off nest intruders (Rollo et al., 2007, Meunier et al., 2009). Round gobies move to deeper waters in the winter (Sapota and Skora 2005, Pennuto et al., 2010). In the spring males swim to the shallow breeding grounds, followed by females (Kotvun, 1976). Females spawn multiple batches through the breeding period (Charlebois et al., 1997).

Receiver sex could influence auditory function in the round goby because male and female round gobies may listen to male acoustic signals for different purposes (mate localization vs. competitive displays). In phonotaxis tests, both males and females show specificity for conspecific acoustic signals relative to heterospecific goby

calls (Rollo and Higgs, 2008). However, females have a higher response rate than males (Rollo et al., 2007). Sexual selection could favour females with greater auditory sensitivity due to the importance of nest localization in female reproductive success. While the behaviour of females between spawning batches is unknown, it has been suggested that females remain at breeding grounds for short periods to deposit eggs, then retreat to deeper waters, potentially to avoid predation (Kotvun, 1979, Young et al., 2010). If so, the effective range of acoustic reception could be greater in females than in males.

Round gobies could also exhibit reproductive state-dependent changes in auditory function due to the seasonal nature of their vocalizations and reproductive behaviour. Their seasonal migration to shallow waters in the spring is similar to that observed by plainfin midshipman fish, a species which exhibits a seasonal shift in auditory sensitivity with the onset of the breeding season (Sisneros and Bass, 2003, Rohmann and Bass, 2011). Additionally, olfactory sensitivity to pheromones in the round goby is flexible depending on reproductive and endocrine condition. In both males and females, olfactory sensitivity to purported pheromones is greater in reproductive fish than non-reproductive fish (Belanger, et al., 2004, Belanger et al., 2007), and is influenced by the level of circulating androgens (Murphy and Stacey, 2002). Since male olfactory and acoustic cues may be sending similar messages - at least to females (Kasurak et al., 2012) - reproductive dependent changes in olfactory sensory physiology could be paralleled by changes in auditory processing.

Chapter 2 Objectives

In Chapter 2, I first examined the effects of sex and reproductive condition on auditory processing in the round goby. In addition, I related plasma 17β -estradiol to hearing measures in females. I also examined the potential for sex and reproductive condition-based differences hair cell densities in the saccule, the main auditory end organ in fishes (Popper and Fay, 1999).

Introduction Part B

Reproductive development in fishes

Teleost fishes exhibit a diversity of reproductive strategies (Wallace and Selman, 1981, Godwin, 2010), which is paralleled by diversity in hormonal mechanisms of gonad maturation and mating behaviour (Kime, 1993, Munakata and Kobayashi, 2010). Due to the potential influence of endocrine and reproductive state on sensory processing and mating behaviour, examining gonadal hormone levels in relation to gonad maturation and mating behaviour is a key step in understanding a species' behavioural endocrinology and neuroendocrinology. Despite the diverse reproductive strategies in fish, many endocrine functions in gonad development are conserved across species (Kime, 1993, Devlin and Nagahama, 2002). The 'classic' models of endocrine control of gonadal development in male and female fishes are described in the following paragraphs.

Reproductive maturation in females is initiated by the release of gonadotropins from the pituitary, which stimulates steroid production in the gonads (Peter and Crim, 1979, Nelson et al., 1990). The steroid 17 β -estradiol (E2) plays a critical role in gonad development by stimulating the liver to produce vitellogenin (Vtg), a glycolipoprotein yolk precursor (Tyler and Sumpter, 1996, Patino and Sullivan, 2002). Vitellogenin is sequestered by the oocytes, resulting in a large increase in egg size. In many female fish studied to date, plasma E2 and testosterone (T) are elevated during vitellogenesis and decline in final maturation (Matsuyama et al., 1991, Kime, 1993). In final maturation and ovulation, steroid production shifts from C19 steroids (androgens, estrogens) to C21 maturation inducing steroids (progestogens) such as 17,20 β -dihydroxy-4-pregnen-3-one (17,20 β -P) (Malison et al., 1994, Munakata and Kobayashi, 2010).

In male fishes, sperm develop in cysts in seminiferous lobules and accumulate in the lumen before collecting in the sperm duct (Grier, 1981). The androgen 11-ketotestosterone (11KT) is considered the primary androgen in many teleosts examined to date (Kime 1993, Borg, 1994, Nagahama, 1994), although there is species variation (Munakata and Kobayashi, 2010). Plasma 11KT has been shown to stimulate spermatogenesis (Miura et al., 1991, Nagahama, 1994), and to decline slightly during spermiation, as steroid production changes to 17,20 β -P (Scott et al., 2010, Schulz et al., 2010).

Evaluating developmental and endocrine correlates of the gonadosomatic index

Gonadosomatic index (GSI) is a commonly used measure of reproductive development in fishes (Tomkins and Simmons, 2002). It measures gonad mass relative

to body mass and can be expressed with the following formula: $\text{gonad mass/body mass} \times 100\%$ (Schreck and Moyle, 1990). In order for GSI to accurately reflect a stage of reproductive development, the increase in gonad mass must correspond to advancement in gamete developmental state; GSI is of little use in distinguishing between stages which contribute minimally to total gonad mass. In mature testes, most of the cell mass may be attributed to an accumulation of spermatozoa (Billard, 1986), and in female ovaries, the main increase in ovary volume can be attributed on an increase in egg size during the vitellogenic phase (Tyler and Sumpter, 1996). Thus, GSI may be used to classify whether fish have reached a spawning capable stage, as indicated by the completion of spermatogenesis or vitellogenesis (Brown-Peterson et al., 2011).

However, patterns of gonad development differ across species and between sexes; therefore, the association between gonad mass and histological stage must be examined on a species-specific basis if GSI is to be used as a proxy for gamete developmental stage. In fish that spawn once in a season, the relationships between gonad developmental stage, endocrine state, and GSI may fluctuate in close association because developmental stages occur in sequence and without repetition of earlier stages. However, in multiple spawning fish, gonad mass may be uncoupled from gonad stage and hormonal condition due to the overlap between mature oocytes and earlier stages of development (Rinchart et al., 1997). In such cases, certain aspects of endocrine state (such as E2 level), may not fluctuate directly with gonad mass because vitellogenesis may be occurring continuously (Rinchart et al., 1996, Rinchart et al., 1997).

Chapter 3 Objectives

In Chapter 3, I examine the covariation between gonad developmental stage, plasma gonadal hormone levels (11KT, T in males, T and E2 in females), and gonadosomatic index (GSI) values. I also describe seasonal changes in GSI and hormone levels in spawning capable fish. The anticipated significance of Chapter 3 was to provide basic knowledge for future studies on the reproductive biology of the round goby. From an applied perspective, understanding the patterns and parameters which determining reproductive activity is a critical research need for management of round goby invasion in the Great Lakes watershed. The results could be used to address the utility of commonly used cutoffs of 1% and 8%, which are now used to classify ‘reproductive’ vs. ‘non-reproductive’ round goby (Gammon et al., 2005, Bowley et al., 2010, Young et al., 2010). There are currently limited data on steroid hormone levels in the round goby in relation to gonadal stage, although hormone levels in different male reproductive groups have recently been reported (Bowley et al., 2010).

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CHAPTER II: CONDITION DEPENDENT AUDITORY FUNCTION IN THE ROUND GOBY, *NEOGOBIUS MELANOSTOMUS*

Introduction

In many animal communication systems, both the production of acoustic signals (e.g. Wells, 1977; Fine, 1978; Kasumyan, 2009) and responses to these signals (Lea et al., 2000; Lynch et al., 2005) vary seasonally in association with reproduction. Signal production and sensory reception traits are likely to be evolutionarily coupled (Endler, 1992). Coupling between sender and receiver traits could also be applied on a temporal scale; when there is seasonal variation in signal production, there could also be potential for seasonal plasticity in auditory function. Seasonal changes in auditory function could be adaptive due to the cost of maintaining sensory neurons (Nivens and Laughlin, 2008). In addition to such condition-dependent effects on audition, sexes typically attend to conspecific acoustic signals with different purpose (e.g. courtship vs. agonistic), which could be reflected by sex differences in auditory function due to differences in auditory tasks (Wilczyński et al., 1984; Searcy and Brenowitz, 1998; Gall et al., 2011).

Effects of season and reproductive condition on acoustic communication are reflected by changes in the neural circuitry involved in vocal production (Tramontin and Brenowitz, 2000; Bass and Zakon, 2005; Bass, 2008) and auditory processing (Bass and Zakon, 2005; Goense and Feng, 2005; Sisneros, 2009; Yoder and Vicario, 2012), which suggests that reproductive state influences the neural mechanisms associated with communication behaviours. Reproductive hormones are likely drivers of the reproductive effects and sex differences in auditory processing due to their close fluctuation with gonadal recrudescence (Kime, 1993; Nagahama 1994), which is

temporally linked with mating in many vertebrates (Nelson et al., 1990; Crews, 1984). Moreover, reproductive hormones have been found to affect vertebrate auditory processing in the midbrain (Goense and Feng, 2005; Maney et al., 2006; Miranda and Wilczyński, 2009) and the auditory periphery (Sisneros and Bass, 2003; Caras et al., 2010).

Many fish show annual patterns of reproductive activity and rely on acoustic communication for mating behaviours (Zelick et al., 1999; Bass and McKibben, 2003; Kasumyan, 2009). Neuroendocrine studies in acoustic and weakly electric fish indicate that endocrine regulation of signal production and reception could be widespread in fish and in other vertebrates (Bass and Zakon, 2005). In female plainfin midshipman fish, *Porichthys notatus*, estrogen and testosterone implantation results in increased phase locking (matching neural spike rate to stimulus cycle) precision at frequencies corresponding to harmonics of male vocalizations (Sisneros and Bass, 2003), and reproductive females have lower auditory thresholds than non-reproductive females caught outside the breeding season (Sisneros, 2009). Similarly, GnRH modulates auditory processing during the reproductive period in the damselfish, *Abudefduf abdominalis* (Maruska and Tricas, 2011). Acoustic communication in teleost fish is a compelling study system for examining the neuroendocrine control of reproduction.

In the current study, I examine condition-dependent auditory plasticity in the round goby (*Neogobius melanostomus*). Several aspects of the breeding biology of the round goby, a vocal benthic teleost, suggest that this species has potential for exhibiting sexual dimorphism and reproductive state-dependent flexibility in auditory function. Reproductive males defend nest territories in rocky crevices (Wickett and Corkum, 1998)

and produce low frequency ‘pulse’ vocalizations (dominant energy \sim 180 Hz) during occupancy (Rollo et al., 2007), which may serve to facilitate female attraction and/or to deter intruders (Rollo et al., 2007; Rollo and Higgs, 2008; Meunier et al., 2009). In playback experiments, females show a stronger propensity to approach acoustic stimuli than males (Rollo et al., 2007), suggesting an overall difference in acoustic behavioural responsiveness between the sexes. Vocal behaviours have only been observed in male round gobies during the breeding season (Rollo et al., 2007), which follows the common observation of minimal or absent vocal communication during the winter in freshwater gobiids (Lugli et al., 1997; Kasumyan 2009). Additionally, Kasurak et al. (2012) found that reproductive females respond more strongly than non-reproductive females to multisensory male olfactory-auditory signals, and olfactory function shows reproductive state-dependent flexibility, with stronger responses in gravid females than non-reproductive females (Belanger et al. 2004; Gammon et al., 2005).

Using auditory evoked potential audiometry (AEPs), I examined the effects of sex and reproductive condition on hearing ability in the round goby. In addition, I related female hearing measures to plasma 17β -estradiol and tested whether hearing differences were related to hair cell density in the saccule, the primary auditory end organ in fish (Popper and Fay; 1999). I measured auditory threshold, suprathreshold response amplitude, and latency of brain responses when presented with tones and a single ‘pulse’ round goby vocalization. I predicted that females would have superior hearing ability (lower thresholds, higher response amplitudes, shorter latencies) relative to males, and that plasma 17β -estradiol level would correlate positively with enhanced auditory phenotypes in females. Within males, I made no specific predictions about the effect of

reproductive condition on hearing ability because while heightened sensitivity to neighbouring male vocalizations could be advantageous for the assessment of neighbouring males, it could also conceivably be unfavourable in that it could increase the propensity to engage in costly agonistic behaviours.

Methods

Subjects

Fish were collected by hook and line from the Detroit River shoreline in Windsor ON Canada between May and August 2010 and 2011. Reproductive fish were tested within 5 days of capture except for 2 females, which became reproductive in the laboratory. Non-reproductive fish were brought into the lab and tested at various dates over the course of the summer. Fish kept in lab were fed fish flakes (Tetramin Inc, Blacksburg, VA, USA) and housed with conspecifics in mixed sex communal tanks on a 16L: 8D photoperiod. Masses and total lengths (TL) (mean \pm s.e.m) of fish used in the study were as follows: non-reproductive females (NRF): mass = 5.96 ± 0.38 g, TL = 8.07 ± 0.19 cm; reproductive females (RF): mass = 6.81 ± 0.47 g, TL = 8.1 ± 0.16 cm; non-reproductive males (NRM): mass = 16.16 ± 3.09 g, TL = 10.81 ± 0.51 cm; reproductive males (RM): mass = 23.11 ± 4.55 g, TL = 12.70 ± 0.93 cm. Details on sample sizes are given in the following sections.

Assessment of reproductive condition

Following hearing tests, fish were euthanized by clove oil overdose and reproductive condition was assessed using a gonadosomatic index (GSI): $GSI = \text{total gonad mass (testes + seminal vesicles)} / \text{total body mass} * 100$ (Schreck and Moyle, 1990). Masses were measured to the nearest 0.01 g (Scout Pro, SP202, Ohaus Corp., Pine Brook, NJ, USA). Males were designated as reproductive when GSI exceeded 1% (Marentette and Corkum, 2008; Bowley et al. 2010) and females were considered reproductive if GSI exceeded 8% (Gammon et al., 2005; Bowley et al., 2010). Gonadosomatic index values in NRFs were (mean \pm s.e.m.): $1.5\% \pm 0.35$ and RFs were $13.2\% \pm 0.94$; in NRMs and RMs they were $0.17\% \pm 0.005$ and $1.6\% \pm 0.12$, respectively. Fish were also sampled for hormone analysis as described below.

Audiometry testing

Auditory stimuli were presented through an underwater speaker (UW-30, Lubell Labs Inc. Columbus, OH, USA) suspended near one end of a PVC cylindrical tank (length = 1.17 m, diameter = 260 mm), which was placed within a sound reducing chamber (Vocalbooth.com, Inc., Bend, OR, USA). Fish were restrained in paper towel without anesthesia on a platform stationed 0.76m away from the speaker at a water depth of 10-12 cm. Sound levels were calibrated before each test day using the maximum cycle RMS value output from an oscilloscope (TDS1002, Tektronix, Inc. Beaverton, OR, USA) connected to a hydrophone (calibration sensitivity – $208.9 \text{ dB re } 1 \text{ V } \mu\text{Pa}^{-1}$, Reson Inc.; www.reson.com).

While pure tone stimuli are useful for evaluating relative sensitivities across frequency ranges, responses to natural calls may elicit different responses than tones (e.g. Maruska and Tricas, 2009; Belanger et al., 2010), and relationships between hearing ability and sex or endocrine status can show stimulus specificity (e.g. Miranda and Wilczyński, 2009; Maruska and Tricas, 2011). Thus, in addition to tones, all fish were presented with a single 96.7 ms pulse (Fig. 1A, B) male vocalization. The stimulus was extracted from a geophone recording of a pulse train from a male round goby defending a nest in the field (Rollo et al., 2007). The pulse stimulus was gated with a 5 ms cosine window and presented at a rate of 4 sec⁻¹. Tone pips were 10 ms in duration and were presented at 100, 200, 300, 400, 500, and 600 Hz. A previous study on round goby hearing revealed that the upper limit of this species' hearing range is approximately 600 Hz (Belanger et al., 2010). The tones were gated with 2 ms Hanning windows and presented at a rate of 8 sec⁻¹. Stimuli were generated using SigGen (v. 4.4) software and AEPs were collected using BioSig (v. 4.4) software (Tucker Davis Technologies, Alachua, FL, USA).

A single stainless steel recording electrode (Rochester Electromedical Inc., Tampa, FL, USA) was inserted under the skin in line with the opercular ridge, and a reference electrode was placed in the snout region (after Belanger et al., 2010). A third grounding electrode was placed into a clay mold which held the fish onto the platform. All stimuli were presented in opposing phases (90° and 270°, 1000 presentations in each phase) with sets of responses from opposing phases averaged to reduce stimulus artifact. Stimuli were initially presented at suprathreshold sound levels and then lowered in 5 dB decrements until responses were no longer observed. During acquisition, responses were

high-pass filtered at 10 Hz, low-pass filtered at 10 kHz, and notch filtered at 60 Hz to remove electrical noise.

Particle acceleration was measured inside the testing tank for all stimuli presented using a triaxial accelerometer (Model 4524, 10 mV ms⁻², Akoustik Engineering Ltd., Windsor, ON, Canada) connected to a conditioning amplifier (Deltatron conditioning amp, model # 2693-A-051. Brüel & Kjær Inc; <http://www.bksv.com>). Accelerometer readings were taken while the device rested on the testing platform in an attempt to mimic stimulation as it would occur at the fish ear. These recordings were taken in another laboratory in an identical tank due to equipment limitations. Acceleration in x, y and z directions were combined into a single measure using the following equation:

$$A = \sqrt{a^2 + b^2 + c^2} \quad (1)$$

Acceleration values were plotted against pressure output from the speaker, and extrapolations from the linear portion of the curve (all R² values were greater than 0.99) were used to obtain acceleration estimates below the noise floor.

Hearing measurements

Threshold, suprathreshold amplitude, and suprathreshold latency were measured offline (Fig. 1C). Threshold was assessed visually as the first obvious waveform deflection from background. Visual methods for threshold assessment have yielded similar results to statistical threshold assessments (Mann et al., 2001; Brittan-Powell et al., 2002, Mooney et al, 2010). Response amplitude and onset latency were measured at 10 dB above threshold to standardize sensation level between animals. Peak-to-peak (pk-pk) amplitude was measured as the difference between the maximum and minimum

voltage collected over the entire recording window, and latency was measured as the time to the trough in the first negative peak of the AEP waveform (Higgs et al., 2003).

Additionally, root mean square (RMS) amplitude was also calculated for pulse responses. This measure was calculated over the 50-150 ms period within the response after performing a 40 Hz high-pass filter with 20dB rolloff per octave.

Sample sizes in hearing analyses varied slightly depending on audiometric measurement and stimulus (tones vs. pulse). Six fish were not presented with the pulse stimulus, and 3 responses to pulses were excluded (2 NRF, 1 NRM) due to significant amounts of electrical noise in the recordings. Additionally, 4 fish (1 NRF, 2 RF, 1 NRM) were excluded from pulse RMS amplitude analyses because the recording window was smaller than the usual 220 ms, cutting off a portion of the response. In these responses, threshold, latency, and pk-pk amplitude could still be measured accurately. Tone amplitude was not measured in 3 fish (3 RF) because the recording window was too short to capture the entire response at 100 and 200 Hz. Sample sizes for tone amplitude and latency responses were lower than for tone thresholds in 6 cases because: (1) at certain frequencies auditory threshold approached the speaker's maximum capacity, preventing acquisition of clean suprathreshold responses, and (2) some fish died near the end of recording, and threshold but not responses at 10 dB above threshold were available.

Despite these differences, the sample sizes in each group were relatively constant across all analyses. The sample sizes for all threshold, pk-pk amplitude, and latency analyses for the pulse stimulus were as follows: 14 NRF, 13 RF, 16 NRM, and 11 RM; sample sizes for the pulse RMS amplitude were: 13 NRF, 11RF, 15 NRM, 11RM. Sample sizes for the tone threshold analyses (see below) were: 13 NRF, 13 RF, 16 NRM,

and 14 RM; sample sizes for the tone amplitude and latency analyses were: 12 NRF, 13 RF, 14 NRM, 11 RM; sample sizes for tone latency were: 12 NRF, 10 RF, 14 NRM, 11 RM.

Plasma 17 β -estradiol assays

Plasma 17 β -estradiol was assayed from 16 females (8 RF, 8 NRF) that had their hearing tested; the sample size was 16 for all tone analyses and 14 for all pulse analyses. After hearing tests, females were anaesthetized with clove oil ($\sim 60 \text{ mg L}^{-1}$) and blood was drawn with heparinized capillary tubes following caudal severance. Blood was spun at 14,500 rpm (Micro-Hematocrit Centrifuge, LWS-M24, LW Scientific, Lawrenceville, GA, USA) for 10 min and then stored at -80°C to be assayed at a later date. Total plasma volumes for fish ranged from 5-40 μL . Prior to assay, steroids were extracted once with diethyl ether and assayed in triplicate using enzyme-linked immunosorbent assays (Cayman Chemical, Ann Arbor, MI, USA). Plasma dilutions for assays were 1:45 and 1:90 for non-reproductive and reproductive females, respectively. Limited plasma volumes precluded running extraction efficiencies on all individuals, therefore extraction recoveries were determined separately for reproductive and non-reproductive females by cold spike recoveries on plasma pools comprised of equal volumes from at least 10 individuals (Bowley et al., 2010). Percent recovery for non-reproductive females was 115.2%, and percent recovery for reproductive females was 95.4%. Inter-assay variability was 11.09%, ($n = 2$), and intra-assay variability was 11.14 ± 13.15 (mean \pm s.d., $n = 46$). Plasma levels were $1.12 \pm 1.25 \text{ ng/mL}$ (mean \pm s.e.m) in non-reproductive females and $2.43 \pm 4.21 \text{ ng/mL}$ in reproductive females.

Sacculle hair cell bundle microscopy

Sacculles were collected from 7 NRF, 8 RF, 8 NRM, and 10 RM in total. Heads were fixed in 4% paraformaldehyde for up to 16 weeks prior to dissection to remove saccular epithelia. Epithelia were stained with Oregon green phalloidin (Molecular Probes Inc., Eugene, OR, USA), which binds to F-actin fibers in stereocilia bundles (Flock et al., 1982). Tissues were soaked for 20 min in a 1:16 dilution [stain:phosphate buffer] (Higgs et al., 2002). Multiple micrograph images were captured across the entire epithelium area at 200x using a fluorescent microscope (Leica DMIRB inverted fluorescent microscope, Wetzlar, Germany). Composite images of the entire epithelium were stitched together using Autostitch (v. 2.2, <http://www.cs.bath.ac.uk/brown/autostitch/autostitch.html>, Ma et al., 2007) under default settings, except that ‘Scale’ and ‘Image Quality’ options were set at 100%. Stereocilia bundles were manually counted from four 10,000 μm^2 boxes distributed across the middle region of the sacculle using ImageJ (v. 1.44, <http://imagej.nih.gov/ij/>) for each saccular epithelium. The locations of these boxes were determined using the following method: (1) a line was drawn that transected the sacculle along the maximum longitudinal distance, (2) seven equidistant lines were drawn perpendicularly along the initial transect line, and (3) counting boxes were drawn over the midpoint of every other one of these seven perpendicular lines, beginning with the line on the most posterior end (Fig. 2).

Statistical analyses

Analyses testing effects of reproductive condition were performed separately within each sex, and sex effects were examined by grouping together reproductive and non-reproductive individuals. Amplitude, latency, and threshold were analysed separately. Mass and length were included as covariates in some analyses when they contributed significantly to statistical models (see below). T-tests were used to examine group differences in pulse responses, and two-way repeated measures ANOVAs were used on tone responses. The repeated measures ANOVAs included stimulus frequency as the within subjects factor and either sex or reproductive condition as the between subject factor. Post-hoc tests were performed using t-tests on averaged responses grouped into low (100-200 Hz) and high (300-600 Hz) frequency categories, with sequential Bonferroni corrections ($\alpha = 0.05/2$ for the lowest p-value) due to the repeated tests on tone responses. The frequency categories represent a distinction between the frequency band corresponding to the dominant energy in round goby male vocalizations (100-200 Hz, Rollo et al. 2007) and higher frequencies, which will propagate further in shallow waters due to the inverse relationship between propagation frequency and water depth (Bass and Clark, 2003). Moreover, AEP traces at 100 and 200 Hz showed distinct slow wave morphologies often observed in low frequency hearing fish AEPs (e.g. Wysocki and Ladich, 2003, Maruska et al., 2007), and are therefore qualitatively different from responses at higher frequencies. Plasma E2 was related to all hearing measures using simple least squares linear regressions. For tonal responses, 17β -estradiol was related to responses grouped into the same low (100-200 Hz) and high (300-600 Hz) frequency averages described above. Variation in hair cell density across epithelial microregions

was tested with a one-way ANOVA. Since no effect of location on hair cell density was observed ($F_{3,95} = 2.20$, $P = 0.09$), data were averaged across boxes. Reproductive condition effects on hair cell density were assessed using t-tests, and ANCOVAs including mass and length were used to test differences between sexes.

Potential influences of body size on hearing ability were examined by including body mass and length as separate covariates. They were removed from models when main effects and interaction terms effects were $p > 0.1$ (Engqvist, 2005). Mass and length contributed to the following models: tone amplitudes within males and between sexes, and pulse threshold within males and between sexes. Mass and length were excluded from analyses which included the following factors: females, latency, tone threshold and pulse amplitude.

Data were assessed for normality using a combination of normality tests (Kolmogorov-Smirnov and Shapiro-Wilk) and probability plots. Hearing data for tone responses were considered normal if most frequency levels (at least 4 of 6) passed formal normality tests. Tone threshold data failed normality tests (presumably due to a lack of sufficient variation), but data fell closely onto the probability plot lines. Plasma 17β -estradiol, length, tone amplitude, RMS amplitude and particle motion threshold were log transformed to meet normality assumptions for hearing analyses, and body mass was log transformed for the saccule hair cell density sex ANCOVA analysis. All statistical tests were performed in SPSS (IBM SPSS Statistics, v. 19.0). Descriptive statistics are reported as mean \pm s.e.m., unless otherwise indicated.

Results

Threshold

Thresholds to the pulse stimulus (120.9 ± 0.068 dB re 1 uPa, $2.75, \pm 0.21 \times 10^{-3} \text{ m s}^{-2}$) were lower than to most tones but similar to responses at 100 Hz (Fig 3). Statistics were not performed to compare tones and the pulse thresholds because these stimuli are not directly comparable due to different RMS power spectra. Audiogram shapes were similar between pressure and particle acceleration audiograms, and as a species, audiograms varied as a function of frequency (*pressure*: $F_{5,275} = 233.57$, $P < 0.001$, m s^{-2} : $F_{5,275} = 46.01$, $P < 0.001$), with lowest thresholds at 100 Hz and highest thresholds in the middle frequencies (300-400 Hz). The maximum threshold in the particle acceleration audiogram was observed at 300 Hz, whereas it occurred at 400 Hz in the pressure audiogram.

Females had lower thresholds to tones than males in both pressure and particle acceleration audiograms, (m s^{-2} : tones: $F_{1,54} = 4.71$, $P = 0.034$, *pressure*: tones: $F_{1,54} = 5.05$, $P = 0.029$), and this effect was frequency dependent (*pressure*: sex*frequency: $F_{5,270} = 2.03$, $P = 0.075$; m s^{-2} : sex*frequency: $F_{5,270} = 5.41$, $P = 0.024$). Post hoc tests revealed that the female effect was restricted to the 300-600 Hz category (*pressure*: $t_{54} = 2.92$, $P = 0.005$, m s^{-2} : $t_{54} = 2.27$, $P = 0.027$) with no significant sex effects at 100-200 Hz (*pressure*: $t_{54} = 0.53$, $P = 0.60$, m s^{-2} : $t_{54} = 0.7$, $P = 0.49$). On average, female thresholds were 2.62 dB ($5.6 \times 10^{-2} \text{ m s}^{-2}$) lower than males at 300-600 Hz. After accounting for mass and length effects using ANCOVA (mass: $F_{1,51} = 3.39$, $P = 0.07$, TL: $F_{1,51} = 1.51$, $P = 0.22$), male and females exhibited no difference in pulse thresholds (mass: $F_{1,51} = 0.32$, $P = 0.57$, TL-sex: $F_{1,51} = 0.70$, $P = 0.41$).

Within females, reproductive state did not affect pulse thresholds ($m s^{-2}$: $F_{1,25} = 1.47$, $P = 0.15$) or tone thresholds ($m s^{-2}$: $F_{1,24} = 0.37$, $P = 0.55$, *pressure*: tones: $F_{1,24} = 0.10$, $P = 0.76$). Auditory thresholds were positively associated with E2 at 100-200 Hz ($m s^{-2}$: $F_{1,14} = 6.06$, $P = 0.027$, *pressure*: $F_{1,14} = 3.53$, $P = 0.081$, Fig. 4A), but not at 300-600 Hz ($m s^{-2}$: $F_{1,14} = 0.28$, $p = 0.61$, *pressure*: $F_{1,14} = 0.23$, $P = 0.64$) or in response to the pulse ($F_{1,12} = 0.46$, $P = 0.51$). For pulse threshold analyses within males, significant reproductive condition*body size interactions (mass: $F_{1,23} = 4.72$, $P = 0.04$; TL: $F_{1,23} = 4.87$, $p = 0.04$) prevented a simple test of main effects. Assessment of the scatterplot indicated no clear elevation between NRMs and RMs, and t-tests revealed no differences between these groups ($t_{25} = 1.39$, $P = 0.18$) (Fig. 5).

Amplitude

Pulse amplitudes ($50.97 \pm 2.77 \mu V$) were greater than amplitudes at most tonal frequencies but similar to responses at 100 Hz (Fig. 6). Again, statistics were not performed to compare responses between tones and the pulse. For tonal stimuli, there was an overall effect of frequency on amplitude ($F_{5,245} = 14.13$, $P < 0.001$), with highest amplitudes at 100 Hz (mean $49.2 \mu V$) and lowest amplitudes at 300 Hz (mean $28.5 \mu V$).

After adjusting for a significant mass effect ($F_{1,44} = 6.82$, $P = 0.012$), there were no differences between sexes in tone amplitude ($F_{1,44} = 0.40$, $P = 0.53$). When length was included as a covariate, there was a sex*length interaction ($F_{1,43} = 4.28$, $P = 0.045$), a sex*frequency interaction ($F_{5,215} = 2.29$, $P = 0.047$), and a sex*frequency*length interaction ($F_{5,215} = 2.40$, $P = 0.038$). An assessment of scatterplots indicated a collinearity problem in that only males were present in the largest length (Fig. 7). To

clarify these interactions, the analysis was run again after excluding the 8 largest fish (2 NRMs, 6 RMs). This removed the length*sex interaction ($F_{1,35} = 0.95$, $P = 0.34$) and the effect of length (ANCOVA, $F_{1,36} = 0.13$, $P = 0.72$) and revealed no sex differences (ANOVA, $F_{1,37} = 3.01$, $P = 0.09$).

Female E2 level was positively related to pulse amplitude (pk-pk: $F_{1,12} = 7.73$, $P = 0.017$, $R^2 = 0.39$, RMS: $F_{1,12} = 3.96$, $P = 0.07$, $R^2 = 0.25$) (Fig. 4B). There was a trend for a positive association between E2 and tone amplitude at 100-200 Hz ($F_{1,14} = 4.49$, $P = 0.052$), but not at 300-600 Hz ($F_{1,14} = 0.002$, $P = 0.96$) (Fig 4C). Female reproductive condition had no effect on pulse amplitude (pk-pk: $t_{25} = 1.61$, $P = 0.12$, RMS (unequal variance t-test): $t_{19,1} = 0.57$, $P = 0.58$) or tone amplitude ($F_{1,20} = 0.31$, $P = 0.58$).

Similarly, there were no differences in pulse amplitude between reproductive and non-reproductive males ($F_{1,22} = 1.80$, $P = 0.19$). After adjusting for mass and length in tone analyses (mass: $F_{1,22} = 7.21$, $p = 0.014$; TL: $F_{1,22} = 5.56$, $P = 0.028$) there were no male reproductive condition effects on tone amplitudes (mass: $F_{1,22} = 2.83$, $p = 0.11$, TL: $F_{1,22} = 2.76$, $P = 0.11$).

Latency

Pulse latencies averaged 49.4 ± 0.23 ms, which was much longer than responses to tones (3.2-17.6 ms), potentially due to the longer rise time of the pulse stimulus relative to tones. There was an overall effect of frequency on tone latency ($F_{5,245} = 299.63$, $P < 0.001$), with longest latencies at 100 Hz (mean = 13.3 ms), and shortest latencies at 600 Hz (mean = 6.3 ms) (Fig. 8). In females, 17β -estradiol level was positively related to pulse latency (Fig. 4D. $F_{1,12} = 5.17$, $P = 0.04$, $R^2 = 0.30$). This hormonal effect was

specific to the pulse stimulus, as 17 β -estradiol was not related to latency at 100-200 Hz ($F_{1,14} = 0.80$, $P = 0.38$) or 300-600 Hz ($F_{1,14} = 0.65$, $P = 0.432$). Reproductive condition had no effect on male pulse latency (male condition: $t_{25} = 0.39$, $P = 0.70$). For tonal stimuli, reproductive males had longer latencies than non-reproductive males ($F_{1,23} = 3.84$, $P = 0.04$). Post-hoc tests revealed that non-reproductive males had shorter latencies than reproductive males at 100-200 Hz (male condition: $t_{23} = 2.77$, $P = 0.011$), but not at 300-600 Hz ($t_{23} = 0.92$, $P = 0.37$). There were no effects of sex or female reproductive condition on latency, neither for the pulse (sex: $t_{52} = 1.32$, $P = 0.19$; female condition: $t_{25} = 0.64$, $P = 0.43$) nor for tones (sex: $F_{1,45} = 0.13$, $P = 0.72$; female condition: $F_{1,23} = 0.05$, $P = 0.82$).

Saccule hair cell bundle densities

Hair cell bundle density did not differ significantly across counting regions ($F_{3,95} = 2.20$, $P = 0.09$), so results were collapsed across microregions, generating a single density measurement for each individual. Males in the sample were longer (t-test: $t_{31} = 8.30$, $P < 0.001$) and heavier (t-test: $t_{31} = 8.51$, $P < 0.001$) than females, indicating a collinearity problem between sex and body size. When assessed with a t-test, females had a higher density of hair cells than males, averaging 13.5 more cells/ 10,000 μm^2 box ($t_{31} = 2.43$, $p = 0.021$), but the sex effect was statistically removed through ANCOVAs including mass and length (mass: $F_{1,30} = 0.73$, $p = 0.40$, length: $F_{1,30} = 0.44$, $p = 0.51$). Both measures of body size, however, did not contribute significantly to the ANCOVAs (mass: $F_{1,30} = 0.40$, $p = 0.53$, length: $F_{1,30} = 0.83$, $p = 0.37$), yet each have significant linear regressions (mass: $F_{1,31} = 6.12$, $p = 0.019$, $R^2 = 0.17$, length: $F_{1,31} = 6.99$, $p = 0.013$, $R^2 = 0.18$). The

relationship between body length and average hair cell density is shown in Fig. 9. As such, it is unclear whether the differences in hair cell bundle density among individuals are due to sex differences or to non-sex specific growth changes. Reproductive condition had no effect on density in either sex (males: $t_{13} = 0.77$, $p = 0.45$; females: $t_{16} = 0.68$, $p = 0.51$).

Discussion

Species hearing ability

The hearing results suggest that the auditory system of the round goby is well equipped for intraspecific communication, which is consistent with behavioural findings that both male and female round gobies show phonotaxis towards conspecific calls (Rollo et al., 2007; Rollo and Higgs, 2008). The dominant spectral peaks in round goby vocalizations typically occur between 100 and 200 Hz (Rollo et al., 2007), and this energy was correlated with auditory responses; the lowest auditory thresholds and highest response amplitudes were observed at 100 and 200 Hz and in response to the pulse stimulus. The audiogram shape in the current study differs from the relatively flat round goby audiogram collected by Belanger et al., 2010, although I similarly observed greatest sensitivity at 100 Hz. The difference between audiograms could be due to the larger number of samples taken during trace acquisition in the current study (1000 in the current study vs. 200 in Belanger et al., 2010). My particle acceleration thresholds are within the range reported for other fish lacking specializations for transducing pressure (Anderson and Mann, 2011). Like other teleosts living in shallow water habitats, the range of

communication in the round goby during breeding season is likely limited to several meters (Mann and Lobel, 1997; Bass and Clark, 2003). However, given that population densities of round gobies colonies can reach more than 10 fish m⁻¹ in rocky substrate (Ray and Corkum, 2001; Johnson et al., 2005), acoustic signals likely function effectively as part of this species' reproductive biology.

Sex differences

I predicted that females could have greater auditory sensitivity than males due to the potentially greater importance of nest localization for mating purposes in females. Lower thresholds in females at higher frequencies could result in sex differences in the effective range of male vocalization detection in shallow waters. Males may nest at depths as shallow as 1 m (Charlebois et al., 1997), which would limit propagation of low frequency components of the vocalization (Bass and Clark, 2003). In propagation of damselfish sounds in shallow water, frequency bands at 400 and 500 Hz had little attenuation at 4 m from the sources, whereas lower frequencies continued to attenuate beyond this mark (Mann and Lobel, 1997). Thus, enhanced auditory sensitivity at 300-600 Hz could facilitate nest localization in females.

There were no effects of mass or length on tone auditory thresholds, which is consistent with Belanger et al.'s, (2010) finding of no differences in auditory sensitivity with size in this species. However, body size had a significant effect on tone amplitude and contributed to variation in pulse threshold. Body size effects were not evident for any hearing measures within females, which could be due to the smaller size range of females. One possible explanation for the body size effects is that mass and length

covary positively with skull casing and muscle tissue above the brain, resulting in a reduced signal from the brain at the recording electrode. In this case, however, it is unclear why mass and length would affect tone amplitude and not tone threshold, and also influence pulse threshold but not pulse amplitude. Additional evidence against the influence of skull size is that the influence of mass on tone amplitude did not appear to be evident at 100 Hz; differences in recording electrode placement should presumably affect all stimuli indiscriminately. Furthermore, hair cell density declined in concert with size, which provides a potential biological explanation for the results.

Although it remains unclear whether the sex differences in auditory function in response to tone amplitude and pulse threshold are due to a non-sex specific growth related effects, they will still have sex-specific consequences. Females typically mature a year earlier than males and are smaller at a given age than males (MacInnis and Corkum, 2000; Young et al., 2010). Additionally, large males are more likely to successfully occupy nests than smaller males (Stammler and Corkum, 2005). Round goby male nest colonies have high densities with relatively low number of aggressive interactions between males (Stammler and Corkum, 2005). A decrease in auditory response could minimize the number of aggressive interactions between conspecifics, which could be one mechanism accounting for high colony densities. This possibility is consistent with observations that reproductive males decrease locomotion and remain in nest in response to olfactory signals from other males (Marentette and Corkum, 2007). It would also resemble a situation in another colonial breeder, the Hawaiian sergeant damselfish *Abudefduf abdominalis*, where GnRH - thought to be released post spawning in this species - has inhibitory responses on auditory thresholds (Maruska and Tricas, 2011).

Latency and amplitude

Latency decreased as frequency increased, and this has been observed in other fish AEP studies (Kenyon et al., 1998; Ladich and Yan, 1998; Higgs et al. 2003). The neural source of origin for specific peaks in transient AEPs are unknown for fishes (Corwin et al., 1982), although earlier waves are thought to correspond to peripheral regions of the auditory pathway (Corwin et al., 1982; Hall, 1992). Some studies have found that delayed auditory latencies are associated with poor auditory function (Lucas et al., 2002; Goense and Feng, 2005; Maruska and Tricas, 2011), and these were the basis of my initial predictions. In AEPs tested on songbirds, shorter latencies were found in the spring, when acoustic communication is most intense (Lucas et al., 2002). In northern leopard frogs, *Rana pipiens*, a shorter first spike latency occurs concurrently with an increase in phase locking (Goense and Feng, 2005). In the Hawaiian sergeant damselfish, *A. abdominalis*, GnRH injection increases first spike latency in the torus semicircularis of damselfish, which occurred in association with increases in threshold (Maruska and Tricas, 2011). On initial assessment this would indicate that long pulse latency in association with E2 level in females and the long latencies in reproductive males at 100-200 Hz are indicative of poorer hearing ability.

However, it is also critical to evaluate latency concurrently with additional hearing measures because they may vary independently. For example, in human ABR studies, increases in auditory brainstem response (ABR) latencies have been associated with high levels of estrogen in the ovulatory phase, which is when females also exhibit greatest auditory sensitivity and frequency discrimination (McFadden, 1998; Walpurger et al.,

2004). Such an enhancement is similar to my results for E2 association with pulse latency and amplitude. The latency shift observed in reproductive males at 100-200 Hz was not associated with a threshold change, so the functional significance of this finding will require further study.

Auditory evoked potential latencies reflect a different measure than individual neuron spike latencies, which should also be considered in the interpretation of the consequence of latency shifts. It is possible that the long AEP latency is associated with a more complex acoustic analysis. The longest AEP latencies are observed at the low tone frequencies in fish, which corresponds to regions of best auditory sensitivity and frequency discrimination. In general, changes in onset latency will have implications for temporal processing of acoustic signals, which could have consequences for pulse repetition rate encoding, a key component of auditory encoding (McKibben and Bass, 2003).

It is similarly difficult to make defined predictions concerning AEP amplitude effects in fish as it is rarely reported (but see Higgs et al. 2003; Wysocki and Ladich, 2003) and represents a clear area in need of further study. Amplitudes of AEP responses should correspond closely with the number of neurons synchronously firing in response to the acoustic stimulus (Hall, 1992), and thus indicate the salience of the acoustic stimulus. Amplitudes of AEPs have been linked to acoustic discrimination in songbirds (Lucas et al., 2002, 2007), although precise measures of frequency selectivity, phase locking, and/or pitch tracking and behavioural studies are required to address these correlates (e.g. Henry and Lucas, 2009). Overall, however, the observed increases in

amplitude in relation to E2 level and the differences between the sexes highlight the importance of auditory reception in females.

Female reproductive condition and hearing

I predicted that reproductive fish would exhibit enhanced auditory function relative to non-reproductive fish due to the greater demands on detection and discrimination of acoustic signals during reproduction. In females, I found that auditory function was similar between females designated as reproductive or non-reproductive, which did not support the prediction. However, circulating E2 level was associated with changes in auditory function, which could indicate that short term hormonal modulation of auditory processing is occurring over the course of the reproductive cycle. For example, in studies on the Hawaiian sergeant damselfish, no effects of sex or season on hearing ability were observed using AEP recordings (Maruska and Tricas 2007), yet administration of GnRH was found to affect auditory processing in the auditory midbrain (Maruska and Tricas, 2011). Also, GSI may not be an accurate measure of reproductive status in female round gobies (Chapter 3), so categorization of “status” may have clouded these effects. Short term hormonal control of auditory function could be an important adaptive mechanism in multiple spawners with a protracted spawning season.

Female plasma E2 level was positively associated with pulse amplitude and latency, but not pulse threshold. While this relationship is associative rather than due to an experimental manipulation, steroids are well known to affect response properties of sensory neurons (Zakon, 1998), and there is increasing evidence for a stimulatory role of steroids in auditory function (Hulcrantz et al., 2002; Bass and Zakon, 2005; Yodel and

Vicario, 2012). The seasonal auditory plasticity in plainfin midshipman is estrogen dependent (Sisneros et al., 2004; Sisneros 2009), although the effects are thought to be due to long term genomic changes in the auditory periphery (Rohmann and Bass, 2011; Coffin et al., 2012). The current study is the first that I am aware of to examine the natural covariation between E2 level and physiological measurement of auditory neural responses in fishes, and its findings are consistent with observations in other taxa, in which E2 has a stimulatory effect on auditory neural responses (Yovanov and Feng, 1983; Tremere and Pinaud, 2009).

During the round goby reproductive cycle, E2 presumably peaks during maximal gonad growth due to the central role of E2 in vitellogenesis (Wallace, 1985; Nagahama, 1994). However, E2 is not an effective trigger of sexual behaviour in female fishes in most species studied to date (Munakata and Kobayashi, 2010) and often decreases during final oocyte maturation (Kime, 1993; Nagahama, 1994). Peak vitellogenic activity in female round gobies could be temporally coupled with migration to shallow waters prior to breeding, and the high circulating E2 levels at this time could affect auditory function to facilitate male localization. Females arrive on the breeding grounds after males (Kotvun, 1979) and may retreat to deeper waters in between spawning batches to avoid predation (Young et al., 2010). However, the associations between E2 and auditory function were mostly related to suprathreshold processing, supporting the notion that E2 affects auditory function at close range. Alternatively, E2 could be produced rapidly in response to vocal stimulation to influence auditory discrimination, as has been observed in other taxa (e.g. birds: Remage et al., 2008; Yoder and Vicario, 2012; anurans: Lynch 2005; Wilczyński and Lynch, 2011). Future studies examining how E2 levels covary

with natural reproductive behaviours and with gonadal development are needed to test these possibilities.

It is interesting that there was a positive association between E2 level and threshold at 100-200 Hz. The effects of hormones and neuromodulators on auditory function are likely to depend on acoustic parameters including frequency content and amplitude envelope (e.g. Miranda and Wilczyński, 2009; Maruska and Tricas, 2009; Maruska and Tricas, 2011). Moreover, the frequencies eliciting maximal neural responses can vary with sound level above threshold (Lu et al., 2004). The pulse stimulus clearly differs from tone responses in its acoustic parameters, and the most salient features relevant for acoustic signal coding in round gobies is currently unknown (but see Rollo and Higgs 2008). One possible explanation for the increase in thresholds at 100-200 Hz is that E2 adjusts frequency filtering properties of the auditory system to increase the critical ratio around vocalization peak frequencies occurring between 100 and 200 Hz. Alternatively, a shift in auditory threshold at the low frequency end could potentially facilitate signal extraction at higher frequencies, where females are more sensitive (300-600 Hz).

Physiological mechanisms

Hair cell densities paralleled hearing performance, were greater in females than in males, and were negatively related to body size. Hair cell numbers increase with age in fishes (Corwin, 1983, Lombarte and Popper, 1994), but ontogenetic studies of hearing in fish have revealed mixed results on changes in auditory sensitivity with growth. Some species exhibit increases in auditory sensitivity with development (Kenyon et al., 1996; Sisneros and Bass, 2005), or no change (Mann et al., 2009), or a decrease (Egner and

Mann, 2005). There are relatively few studies that have directly related hair cell number with auditory ability (Corwin et al., 1983; Higgs et al., 2001; Coffin et al., 2012). In a study on the elasmobranch ray, *Raja clavata*, for example, an increase in total hair cell number was associated with an increase in auditory sensitivity (Corwin et al., 1983). In contrast, Higgs et al. (2001) found no change in auditory sensitivity with hair cell addition in zebrafish (*Danio rerio*).

While an increase in total hair cell number may increase the amount of information travelling in the auditory nerve, the corresponding growth of the rest of the brain and other auditory structures (e.g. otolith, swim bladder, swim bladder connections) could influence the significance of hair cell addition on auditory function. For example, some authors have argued that the addition of hair cells may maintain auditory threshold as the otolith grows and the distance between swim bladder and ear increases (Higgs et al., 2002, 2003). Thus, hair cell density may interact with other factors to affect auditory function; a simple relationship between hair cell density and auditory sensitivity may not always be found.

Nonetheless, in the current study, the decline in hair cell density with increasing length corresponded to a decline in hearing ability. Changes in hair cell number could be one possible mechanism for the sex differences in auditory sensitivity observed in the current study. In plainfin midshipman, a fish lacking pressure detection specializations, auditory sensitivity is positively related to hair cell bundle density; however, hair cell bundle density is negatively related to size in females (Coffin et al., 2012). Hair cell number may show tighter links with auditory sensitivity in fish lacking pressure detection

specializations, since the allometric growth of auditory specializations does not need to be considered.

17 β -estradiol could affect auditory function through several possible mechanisms. Estrogen receptors have been found in the saccule of plainfin midshipman (Forlano et al., 2005) and the African cichlid (*Astatotilapia burtoni*) (Maruska and Fernald, 2010), suggesting that E2 could affect auditory sensitivity and frequency filtering in the auditory periphery. 17 β -estradiol may influence hair cell ion channel production and kinetics. The number and kinetics of hair cell ion channel activation and deactivation is a main determinant of hair cell resonance (Fettiplace and Fuchs, 1999), which could be a major factor in determining the frequency filtering properties of the auditory system. Changes in ion channel function could occur through genomic or non-genomic mechanisms. Steroid hormones are capable of inducing transcription of ion channels (Zakon, 1998; Few and Zakon, 2007), but E2 can also rapidly (within seconds) inhibit K⁺ channels in stria vascularis in gerbils (Lee and Marcus, 2001), indicating non-genomic action. Alternatively, since AEP measures the whole brain response, E2 could affect auditory function in the midbrain; E2 receptors have been found in the auditory midbrain of anurans (Chakraborti and Burmeister, 2010).

Conclusions

The current results reveal sexual dimorphism in auditory function in a novel teleost system and the potential for control of auditory function through E2 and hair cell number. This contributes to a framework for future studies on the ecological conditions and

physiological mechanisms of condition-dependent and sexually dimorphic auditory function. Future studies examining the relationships between reproductive endocrine status and behavioural responses to acoustic signals are needed to place these results in an ecological context.

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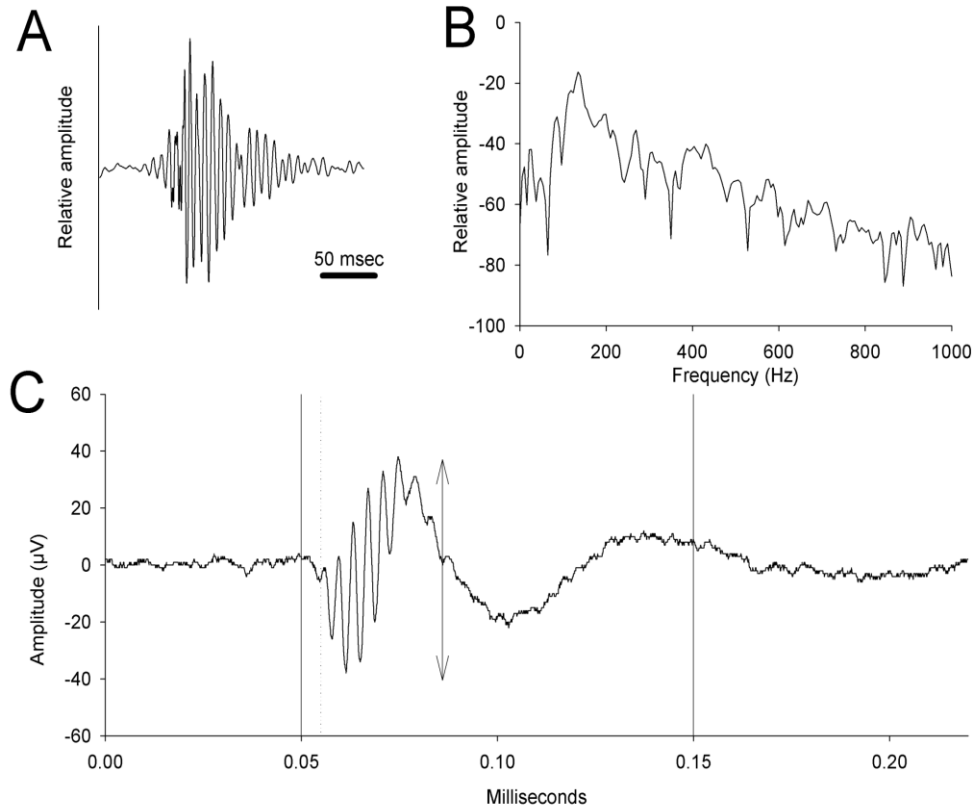
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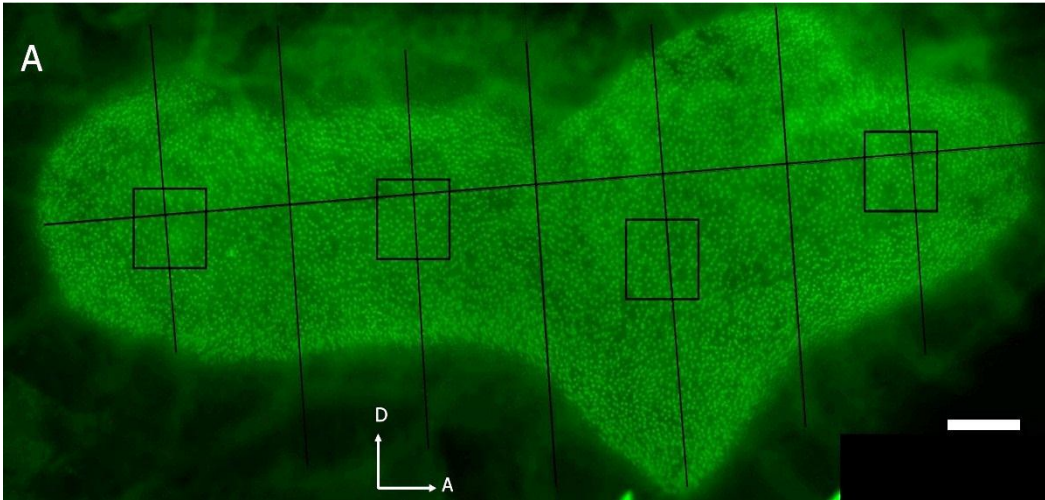
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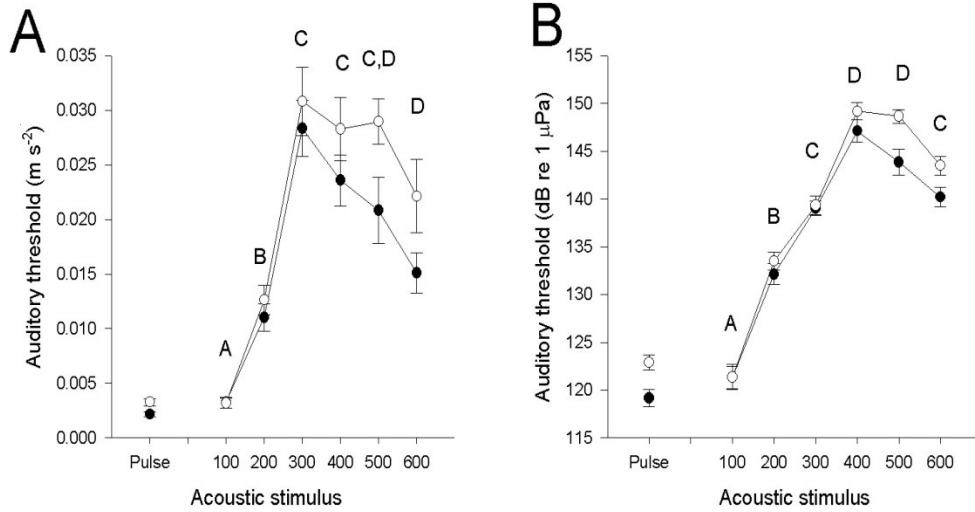
Figures



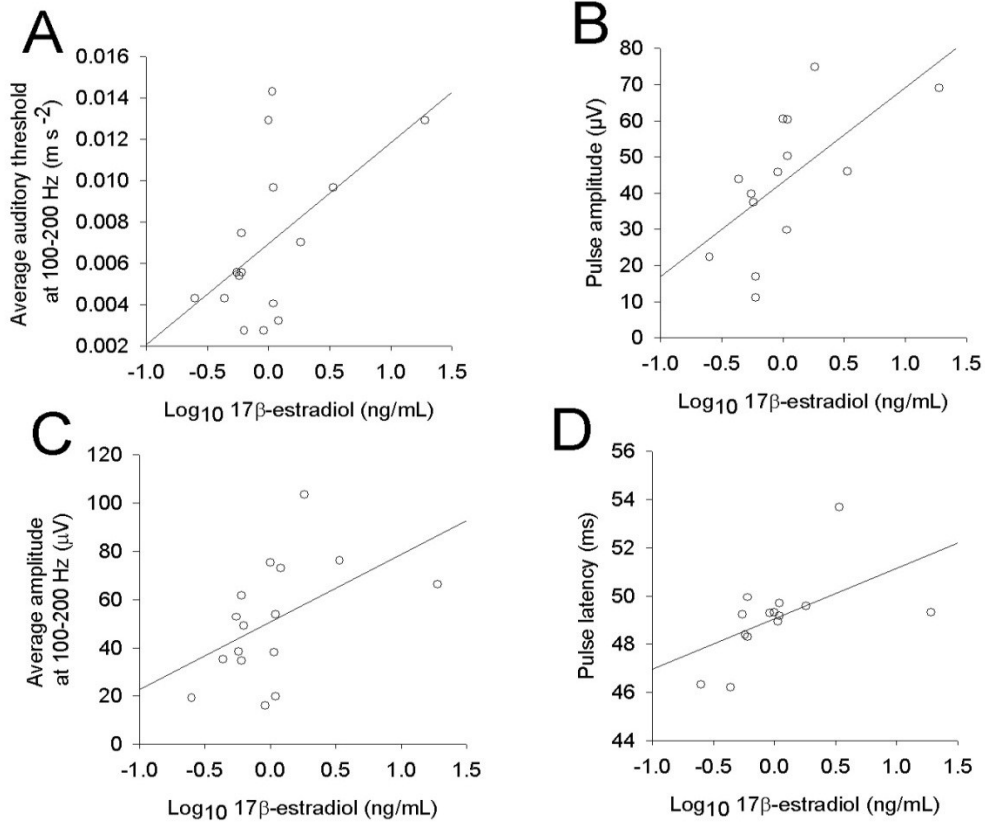
Chapter 2, Fig. 1. Waveform of a single male round goby pulse stimulus recorded from a hydrophone at the location of the recording platform in the experimental tank. (B) Fast Fourier transform (8192 pt Hanning window) created over 9 seconds of pulse repetition in tank. (C) A representative AEP response elicited in response to the pulse stimulus. The onset latency peak is indicated with a dotted line and peak-to-peak amplitude measurement is indicated with a double ended arrow. The region of the stimulus used for RMS amplitude measurement is denoted with solid lines at 50 and 150 ms.



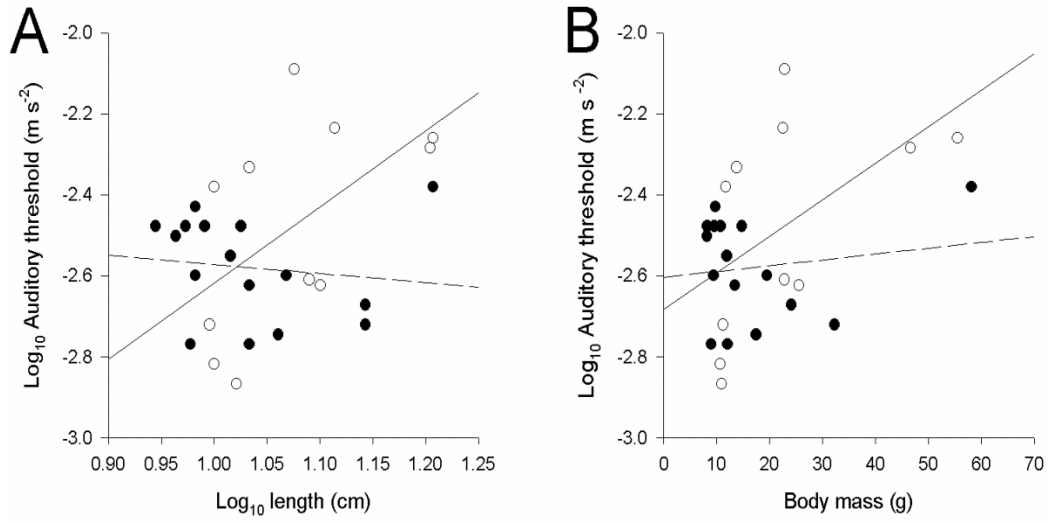
Chapter 2, Fig. 2. (A) Line transects and distribution of the four counting regions of phalloidin labeled hair cell bundles in the saccule (see text for details). Each box covers $10,000\mu\text{m}^2$. Methods for box placement are described in the text. D = dorsal, A = anterior. Scale bar represents $100\mu\text{m}$.



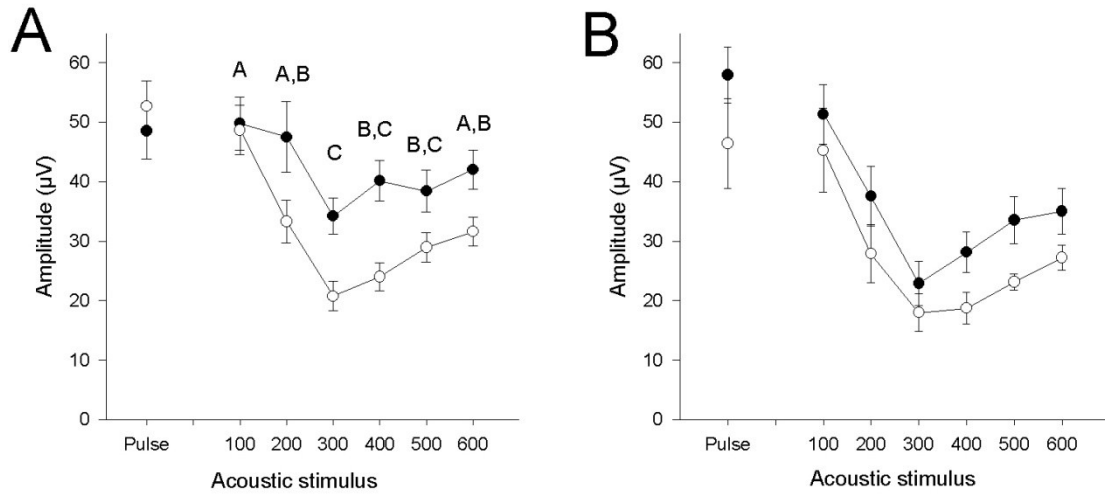
Chapter 2, Fig. 3. Auditory threshold in males (open circles) and females (closed circles) in response to the ‘pulse’ and tones, expressed in (A) pressure, and (B) particle motion. Error bars are mean \pm s.e.m. in all hearing measure figures. Letters above tone responses indicate results of Tukey HSD post hoc tests ($\alpha = 0.05$) following a species-wide (males and females combined) test of frequency on auditory threshold. Frequencies possessing different letters are significantly different.



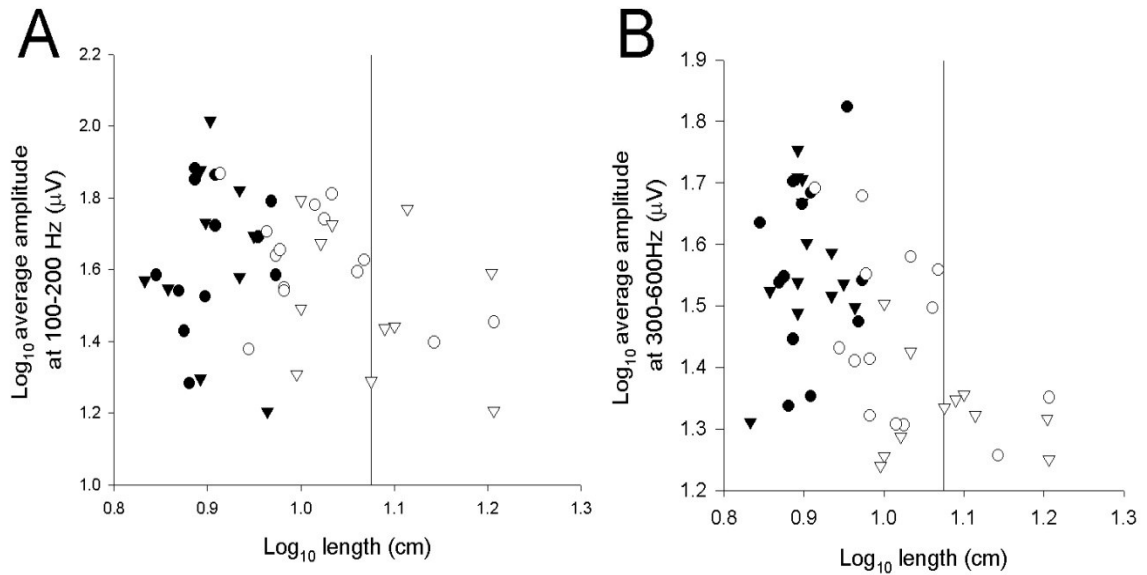
Chapter 2, Fig. 4. Linear regressions of female 17 β -estradiol level on (A) average particle motion threshold (m s^{-2}) between 100 and 200 Hz, (B) pulse response peak-to-peak amplitude, (C) average amplitude at 100 and 200 Hz, and (D) pulse onset latency. Each data point in A and C represents a subject's average response between 100 and 200 Hz (see text for details).



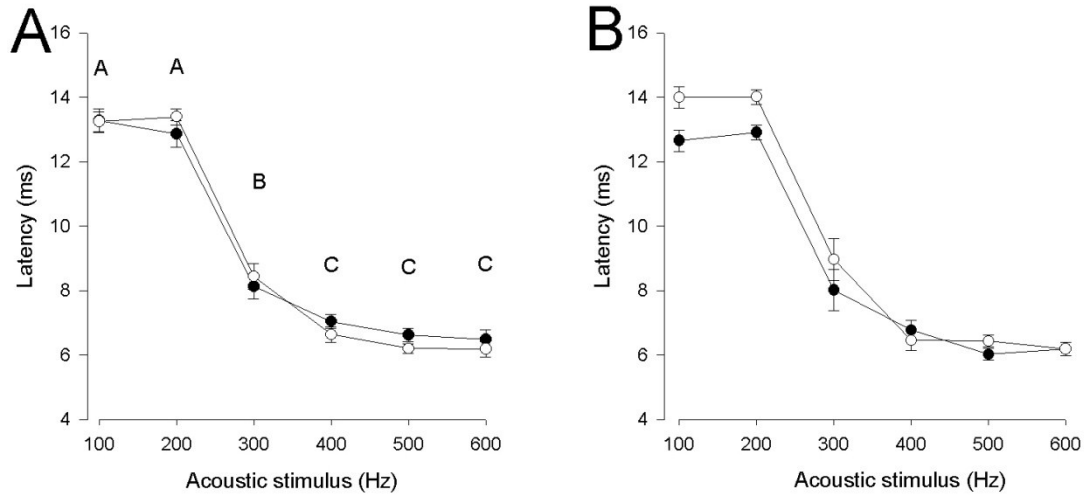
Chapter 2, Fig. 5. Scatterplot illustrating the interactions between male length (A) and body mass (B) in relation to pulse auditory threshold. Lines indicate separate regressions performed for non-reproductive males (dashed line, filled circles) and reproductive males (solid line, open circles).



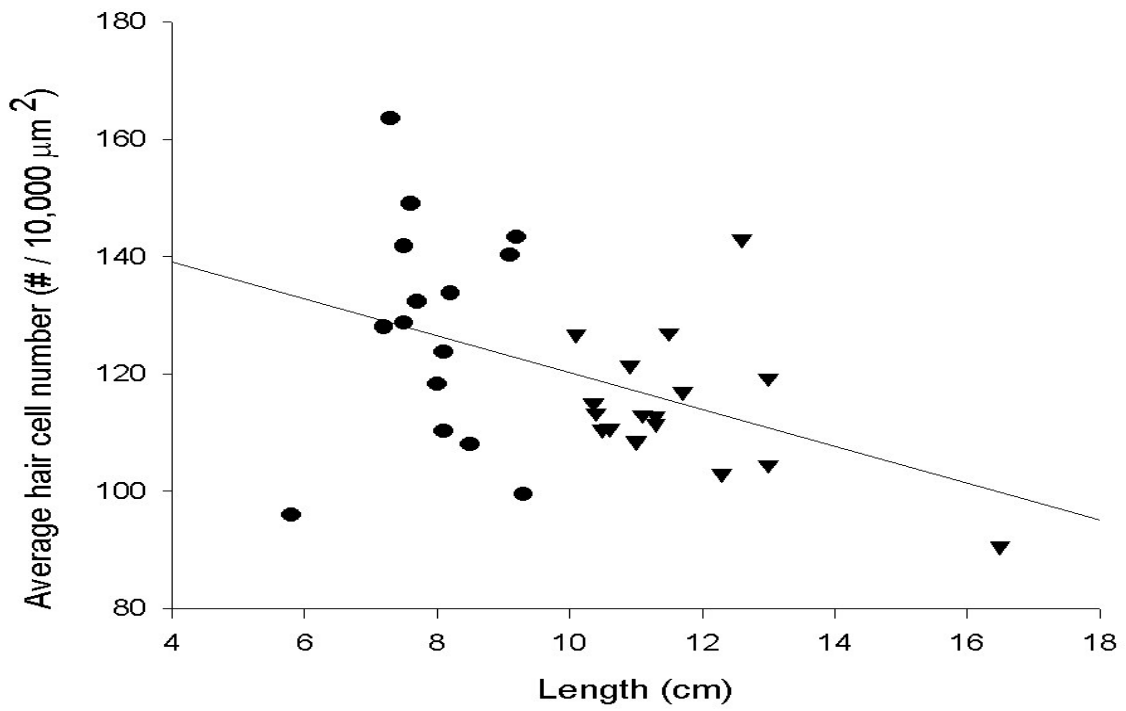
Chapter 2, Fig. 6. Peak-to-peak amplitudes in response to the pulse and tonal stimuli grouped by sex (males = open circles, females = closed circles). (B) Male peak-to-peak amplitudes grouped by reproductive condition (reproductive males = open circles, = non-reproductive males = closed circles). Letters above tone responses indicate results of Tukey HSD post hoc tests ($\alpha = 0.05$) following a species-wide (males and females combined) test of frequency on response amplitude. Frequencies possessing different letters are significantly different.



Chapter 2, Fig. 7. Scatterplot illustrating the association between length and tone amplitudes at 100-200 Hz (A) and 300-600 Hz (B) in all reproductive groups (non-reproductive females = black circles, reproductive females = black triangles, non-reproductive males = open circles, reproductive males = open triangles). Each data point represents an average of responses to all frequencies within the respective categories. The vertical lines separate the 8 largest fish (males) removed from the sex ANCOVA analysis (see text for details).



Chapter 2, Fig. 8 (A) Onset latency in response to tonal stimuli grouped by sex (males = open circles, females = closed circles). (B) Male peak-to-peak amplitudes grouped by reproductive condition (reproductive males = open circles, = non-reproductive males = closed circles). Letters above tone responses indicate results of Tukey HSD post hoc tests ($\alpha = 0.05$) following a species-wide (males and females combined) test of frequency on response latency. Frequencies possessing different letters are significantly different.



Chapter 2, Fig. 9. Linear regression of total length on average hair cell number per 10,000 μm^2 counting region (females = circles, males = triangles).

CHAPTER III: ASSOCIATIONS BETWEEN GONADOSOMATIC INDEX, GONADAL STEROIDS, AND GONAD DEVELOPMENT IN MALE AND FEMALE ROUND GOBIES, *NEOGOBIUS MELANOSTOMUS*

Introduction

Accurately assessing reproductive condition is an important task in fisheries management and fish biology research. The gonadosomatic index (GSI), given by the equation: $(\text{gonad mass} / \text{body mass}) \times 100\%$ (Schreck and Moyle, 1990), is a commonly used metric to determine reproductive condition in fishes (deVlaming et al., 1971, Tomkins and Simmons, 2002). The GSI is an easy metric to collect and can correlate well with gonadotropins and gonadal steroid hormones (Campbell et al., 2006, Schulz, 2010, Lubzens et al., 2010) and stage of reproductive development. The GSI is also commonly used to document seasonal trends in reproduction (Scott et al., 1980, Prat et al., 1990, Rinchard et al., 1993, Sisneros et al., 2004).

The utility of the GSI as a measure for reproductive condition, however, must be examined on a species-specific basis because it depends on the pattern of gonad development (Rinchard et al., 1996, Lowerre-Barbieri et al., 1996). While GSI may be a reliable indicator of gonad development in group synchronous spawners (Modesto and Canario, 2003, Sisneros et al., 2004), it provides less reliable information on reproductive status in species that spawn repeatedly throughout the breeding period, particularly if the breeding season is extended. In multiple spawning species, fish that have already spawned at least once may display similar GSI values to fish developing their first batch (Rinchard et al., 1996, Lowerre-Barbieri et al., 1996). Additionally, steroid sex hormonal profiles across the reproductive cycle may differ between single spawners and multiple spawners due to the potential concurrent production of a subsequent batch (Rinchard et

al., 1997). Using GSI alone to evaluate reproductive condition in multiple spawners could mask features of the animal's reproductive condition and could affect conclusions on the behaviour and physiology of "non-reproductive" subjects.

Round gobies (*Neogobius melanostomus*), an invasive species in the Laurentian Great Lakes (Charlebois et al., 2001, Corkum et al., 2004), are multiple spawners (Charlebois et al., 1997, Kornis et al., 2012) and it has been suggested that this trait has contributed to their invasion success (MacInnis and Corkum, 2000, Charlebois et al., 2001). Round gobies have a long breeding season (May-October, Young et al., 2010), although spawning intensity is greatest in May and July (MacInnis and Corkum, 2000, Young et al., 2010) and the length of the season depends on water temperature (Moiseyeva and Rudenko, 1969, Charlebois et al., 1997). Females lay 3-6 batches over the course of the summer (Charlebois et al., 1997, Kulikova, 1985, MacInnis and Corkum, 2000) in nests guarded by males (Charlebois et al., 1997). After spawning, female GSI decreases to an average of 1.8% at 6 days after spawning (Kulikova 1985).

The use of the GSI has now become a common metric of assessment of reproductive state among researchers interested in round goby biology (Gammon et al., 2005, Marentette and Corkum, 2008, Marentette et al., 2009, Bowley et al. 2010, Yavno and Corkum, 2010, Young et al., 2010), yet there exists little data on the validity of this metric or its correspondence with hormonal state or gonad development (but see Bowley et al. 2010). The goal of the current study was to examine the associations between sex hormone status, gonadal stage, and GSI in round goby. Additionally, seasonal changes in GSI and gonad hormones were examined in reproductive fish to assess the potential for these metrics to show seasonal fluctuation.

Methods

Subjects

Round gobies were collected by angling from the shoreline of the Detroit River between April and August 2011. Reproductive males and reproductive females were processed within one week of capture, whereas non-reproductive fish were euthanized after being held in the lab for various lengths of time. Lab fish were housed in aquaria with conspecifics in mixed sex tanks and fed with fish flakes (Tetramin Inc, Blacksburg, VA, USA) and kept on a 16L:8D photoperiod.

Gonadosomatic index (GSI) was calculated using the following equation: (gonad mass /body mass) \times 100% (Shreck and Moyle, 1990). Masses were measured to the nearest 0.01 g (Scout Pro, SP202, Ohaus Corp., Pine Brook, NJ, USA). Round goby sex in adults is easily determined externally by urogenital shape (Charlebois et al., 1997). Males with a GSI greater than 1% were considered “reproductive” (“RMs”) (Marentette and Corkum, 2008, Marentette et al., 2009, Bowley et al. 2010, Young et al., 2010), and females with a GSI greater than 8% were considered “reproductive” (“RFs”) (Gammon et al., 2005, Yavno and Corkum, 2010, Bowley et al., 2010). Masses and total lengths (TL) (mean \pm s.e.m) in each reproductive group, as determined by GSI cutoffs were as follows: non-reproductive females (“NRF”): mass: 6.4 ± 0.60 g, TL: 8.14 ± 0.18 cm; reproductive females (“RF”): mass: 6.83 ± 0.38 g, TL = 8.05 ± 0.17 cm; non-reproductive males (“NRM”): mass: 15.33 ± 1.79 g, TL: 10.75 ± 0.32 cm; reproductive males (“RM”): mass: 20.44 ± 2.5 g, TL: 11.76 ± 0.38 cm. No “sneaker males”

(Marentette et al., 2009) were included in this study. Average GSI (mean \pm s.e.m) in each group were as follows: “NRF”: $1.9 \pm 0.27\%$, “RF”: $12.9 \pm 0.59\%$; “NRM”: $0.15 \pm 0.029\%$; “RM”: $1.8 \pm 0.10\%$.

Plasma steroid assays

Males were tested for 11-ketotestosterone (11KT) and testosterone (T) levels, and females were tested for 17 β -estradiol (E2) and testosterone. Following anaesthesia with clove oil ($\sim 60 \text{ mg L}^{-1}$), blood was drawn from the caudal vein using heparinized capillary tubes, following caudal severance. The blood was spun at 14,500 rpm (Micro-Hematocrit Centrifuge, LWS-M24, LW Scientific, Lawrenceville, GA, USA) for 10 min and then stored at -80°C and assayed at a later date. Total plasma volumes for fish typically ranged from 5-40 μL . Some plasma samples were collected after a behavioural sensory experiment involving round goby acoustic or olfactory signals, or an auditory physiology test (Chapter 2). Differences in hormone levels in these fish relative to fish collected directly from lab were tested (see below). All plasma collection was done in the afternoon between 13:00 and 18:00 h to account for possible diel influences on hormone levels.

Steroids were extracted once with diethyl ether prior to assay. Samples were run in triplicate using enzyme-linked immunosorbent assays (Cayman Chemical, Ann Arbor, MI, USA) with individuals randomly assigned to plates. Inter-assay variabilities, calculated from a single control triplicate across plates, were 41.8% (n = 3), 29.10 % (n = 4), and 11.14%, (n = 2) for 11KT, T, and E2, respectively. Intra-assay variabilities were $5.22\% \pm 8.13$ (n = 58), 5.16 ± 6.09 (n = 96), and 11.14 ± 13.15 (n = 46) for 11KT, T, and

E2, respectively. The total number of individuals samples for steroids in each group were as follows: “NRF”: 22 T, 22 E2; “RF”: 17 T, 19 E2; “NRM”: 25 11KT, 23 T; “RM”: 19 11KT, 18 T.

Limited plasma volumes precluded running extraction efficiencies on all individuals. Therefore, extraction recoveries were determined separately for each reproductive group by cold spike recoveries on plasma pools comprised of equal volumes from at least 10 individuals (Bowley et al., 2010). Recoveries for the different reproductive groups are summarized in Table 1, and additional information on calculations are provided in the Appendix. Extractions of testosterone were sub-optimal and differed between reproductive groups, which were considered for data analysis and interpretation (see below). Despite different dilution and spike amounts, similar recoveries between 11KT and T were observed for the same plasma pools in males.

Gonad histology

Following GSI morphometric measurements, gonadal tissue was placed in 10% formalin (after Marentette et al., 2010) and transferred within three months to 70% ethanol for long term storage before performing histology (2-8 months). Testes were dehydrated, cleared with xylene, embedded in paraffin wax (Fisher Ltd.), and sectioned at 5-7 μm using a microtome (Leica RM2125, RT, Leica Microsystems Nussloch GmbH, Nussloch, Germany). Vitellogenic ovaries commonly burst during the dehydration and clearing process for paraffin embedding, so they were embedded in a glycol methacrylate resin immuno-bed kit (Model #17324, Polysciences, Inc., Warrington, PA) and sectioned at 10 μm . Large gonads were cut in half along the medial-lateral axis before embedding.

Multiple sections were taken near the middle of each gonad. Testis and ovary sections were mounted on Superfrost Plus slides (VWR International, LLC, Radnor, PA), placed on a slide warmer with water to facilitate slide adherence, and stained with hematoxylin and eosin. Sections were viewed under a light microscope (Leica DME, Buffalo, NY). Gonad developmental staging was based on criteria proposed by Brown-Peterson et al., (2011). Criteria are described in Table 2, and representative light micrographs of ovaries and testes in each stage are shown in Fig. 1 and Fig. 2, respectively.

Statistical analyses

Effects of collection method on hormone level were performed due to the potential influence of handling stress (Pickering et al., 1987) and/or social stimulation from conspecific signals from previous tests on hormone levels (Oliviera et al., 2001, Remage-Healey and Bass, 2005). Within each GSI group, t-tests were performed to compare hormone levels from fish that were sampled directly from lab to samples that were taken following a behaviour or hearing test. Samples from the two latter ‘indirect’ collection methods were pooled together before the test due to small sample sizes. “Reproductive females” were excluded from these analyses because only two were collected directly from the laboratory. However, the sample sizes of hearing and behaviour tests in “RFs” were large enough to test for possible differences between hearing and behaviour tests on hormone levels, which was tested with a t-test.

T-tests were used to compare hormone levels between GSI groups within each sex, and omnibus adjustments were made to 100% based on the mean recoveries, and then tested between reproductive groups. Due to the potential confounding effect of

extraction efficiencies on the hormone data, all additional analyses including androgen data were performed within each GSI group. To examine the hormonal milieu, Pearson correlations were performed between E2 and T in females and between 11KT and T in males. Gonadosomatic index was related to hormone levels using Pearson correlations. Since all “RMs” and no “NRMs” were classified into the ‘spawning capable’ stage based on gonad staging (see Results), analyses comparing androgen levels between the different gonad stages were restricted to “NRMs”. Comparisons of androgen levels within “NRMs” were further reduced to t-tests between immature and developing males due to the exclusion of a small sample size of males in the regressing stage (n = 3). Within females, all “RFs” were in the spawning capable stage, whereas most “NRFs” were in the developing stage (see Results). Thus, an explicit test of differences in T levels within each reproductive group was not done due to small sample sizes. Plasma E2 data (pooled across “NRF” and “RF” groups) were compared between the spawning capable and developing groups using t-tests.

Seasonal variability in GSI and reproductive hormones was examined with scatterplots relating ordinal date to these parameters. Scatterplot analyses were restricted to “RMs” and “RFs”, because these fish were always processed within a week of capture. All data were examined for normality using probability plots and Kolmogorov-Smirnoff tests. Hormone data were log transformed to meet normality assumptions.

Results

Histological classification

No immature females (only oogonia present) were observed, and the vitellogenic egg cohort occurred alongside primary growth and cortical alveolar oocytes (Fig 1B,C). Based on ovarian histology, all “RFs” possessed eggs with complete vitellogenesis. In “NRFs”, 13/17 were in the developing stage and 4/17 were in the spawning-capable stage. Within the developing stage, 10/13 were actively vitellogenic, whereas 3/13 had cortical alveolar oocytes as the most advanced stage. In the three spawning capable “NRFs”, although yolk was contained throughout the cytoplasm, the oocytes were likely not undergoing final maturation, as indicated by a relatively thin follicular layer. All “RMs” (16/16) possessed spermatozoa within the lobule lumen (spawning capable), whereas no “NRMs” were classified as spawning capable. Within “NRMs”, 8/21 were in the immature stage, 10/21 were in the developing stage, and 3/21 were in the regressing stage. A summary of the numbers and GSI in each category is shown in Table 3.

Hormone levels and GSI

In all hormones and all sex groups tested, fish that were collected directly from lab tanks exhibited similar hormone levels to fish that were exposed to a behavioural or hearing tests (“NRMs”, 11KT: $t_{22,9} = 0.4$, $p = 0.69$; T: $t_{8,8} = 0.60$, $p = 0.56$, unequal variance t-test; “RMs”, 11KT: $t_{6,28} = 1.5$, $p = 0.18$, unequal variance test, T: $t_{5,21} = 1.6$, $p = 0.17$ unequal variance t-test; “NRFs”: E2: $t_{20} = 0.18$, $p = 0.86$; T: $t_{12,3} = 0.16$, $p = 0.88$,

unequal variance t-test). Within “RFs” there was no difference in hormone levels after behavioural tests versus hearing tests (E2: $t_{15} = 0.85$, $p = 0.41$; T: $t_{13} = 0.45$, $p = 0.81$).

Plasma E2 and T levels were positively correlated in females ($r = 0.52$, $p = 0.003$), and the positive correlations remained when GSI groups were evaluated separately (NRFs: $r = 0.75$, $p = 0.001$; RFs: $r = 0.54$, $p = 0.036$) (Fig 3A). Plasma E2 values were similar between “NRFs” (1.1 ± 1.3 ng/mL) and “RFs” (2.4 ± 4.2 ng/mL; $t_{39} = 1.43$, $p = 0.17$). There was no overall correlation between E2 and GSI in females ($r = 0.11$, $p = 0.48$, Fig 4A). When examined within each GSI group, E2 was not related to GSI (“NRFs”: $r = 0.38$, $p = 0.082$; “RFs”: $r = -0.39$, $p = 0.10$) (Fig. 5A). Plasma E2 levels were similar between developing and spawning capable stages ($t_{26} = 0.014$, $p = 0.99$). Female testosterone levels were higher in “NRFs” (11.4 ± 12.8 ng/mL) than “RFs” (6.3 ± 7.2 ng/mL; $t_{37} = 2.37$, $p = 0.023$) (Fig 5B). However, it is likely that the reported difference in testosterone levels between “NRFs” and “RFs” was introduced by the correction needed for low extraction efficiencies in “NRFs”. Testosterone level was positively associated with GSI value in “NRFs” ($r = 0.574$, $p = 0.005$), and negatively associated with GSI in “RFs” ($r = -0.634$, $p = 0.006$, Fig. 4B).

Male 11KT and T level were positively associated with each other ($r = 0.69$, $p < 0.01$), and when evaluated within each GSI grouping, their levels were similarly positively correlated (“NRMs”: $r = 0.50$, $p = 0.012$, “RMs”: $r = 0.59$, $p < 0.01$) (Fig. 3B). 11-ketotestosterone levels in “RMs” (30.9 ± 7.6 ng/mL) were higher than “NRMs” (6.7 ± 1.7 ng/mL; 11KT: $t_{42} = 6.12$, $p < 0.01$) (Fig 5C). Similarly, levels of testosterone were higher in “RMs” (5.26 ± 0.96 ng/mL) than “NRMs” (2.3 ± 0.3 ng/mL, T: $t_{39} = 4.50$, $p < 0.01$, Fig 5D). Gonadosomatic index was not correlated with 11KT or T levels in

“NRMs” (11KT: $r = 0.30$, $p = 0.248$, T: $r = 0.225$, $p = 0.42$) or “RMs” (11KT: $r = 0.2$, $p = 0.42$; T: $r = 0.02$, $p = 0.94$) (Fig. 6). Within “NRMs”, there was a trend for developing males to have higher 11KT levels than immature fish ($t_{16} = 2.03$, $p = 0.059$, Fig 7A), but testosterone levels were not different between these groups ($t_{14} = 0.60$, $p = 0.56$, Fig 7B).

Seasonality

Females with oocytes in late vitellogenic and final maturation stages (GSI > 8%) were found throughout May-August. The overall investment appeared to decline (GSI), although hormone levels showed no clear trends. In females there was a trend towards a decrease in GSI as the season progress, but no changes in E2 or T levels (Fig. 8A,C,E). Similarly, males with sperm (GSI > 1 %) were found between May and August. Female testosterone levels ranged from 1.6 ng/mL to 24.3 ng/mL, while E2 ranged from 0.082 ng/mL to 19.0 ng/mL. In males, there were no evident seasonal changes in GSI, 11KT, or T (Fig 8B,D,F). Testosterone levels ranged from 2.33 to 18.65 ng/mL, while 11KT levels ranged from 6.46 to 145.05 ng/mL.

Discussion

Methodological considerations

The extraction recoveries in androgens were sub-optimal and showed variation between reproductive group plasma pools. Therefore, androgen levels should not be considered as absolute, and readers are referred to Bowley et al., 2010 for levels in different male reproductive groups. While this indicates the presence of unexplained

variation in the steroid hormone data, the results contain several patterns which are consistent with biological expectations (see discussion below). Additionally, comparisons between each GSI group using the omnibus adjusted values match expectations for relative differences between reproductive groups in male round gobies, as reported by Bowley et al. (2010). One exception, however, is that while Bowley et al., (2010) found no differences in T levels between “RMs” and “NRMs” I found greater T in “RMs” relative to “NRMs”.

Females

The results indicate that the common practice of assigning female reproductive status based on the 8% cutoff alone (Gammon et al., 2005, Marentette and Corkum, 2008, Marentette et al., 2009, Bowley et al. 2010, Yavno and Corkum, 2010, Young et al., 2010), can result in misleading classifications. Gonadosomatic index values mask the high degree of variation in female endocrine condition and gonad stage. “Non-reproductive females” commonly possess vitellogenic oocytes, can have late stage vitellogenic oocytes, and can have relatively high steroid hormone levels. Thus, it is possible that oocyte maturation may occur at a smaller total gonadal volume than previously thought. As defined by Brown-Peterson et al. 2011, the spawning capable stage describes the completion of vitellogenesis, rather than the completion of final oocyte maturation (germinal vesicle breakdown). However, the receptors necessary for oocyte maturation should be present at the end of vitellogenesis (Brown-Peterson et al., 2011).

While E2 and T did not show simple linear correlations with GSI across the entire range, and no overall differences between “NRFs” and “RFs”, they still likely show some variation across the reproductive cycle, since vitellogenic round goby oocytes develop as distinct cohorts (Kulikova 1985). The highest levels of E2 were observed in mid-GSI ranges, which could indirectly approximate peak vitellogenic activity due to the role of E2 in stimulating vitellogenesis (Matsuyama et al., 1991, Tyler and Sumpter, 1996, Campbell et al., 2006). Additionally, the positive relationship between GSI and T in “NRFs” could indicate the positive relationship between steroid production and oocyte growth. Testosterone and E2 are commonly elevated together during gonadal recrudescence in both multiple and total spawning fish (Methven et al., 1992, Rinchard et al., 1998, Mandich et al., 2004, Sisneros et al., 2004) and their levels correlate with gonadal growth during vitellogenesis (Campbell et al., 2006).

The relatively constant steroid levels between developing and spawning capable fish is consistent with Bowley et al.’s (2010) observation of similar vitellogenin mRNA expression rates between “NRFs” and “RFs”, based on the same GSI cutoffs used in the current study. The stable E2 levels across GSI levels could also be explained by the presence of cortical alveolar stage oocytes throughout the developing batch. The onset of the cortical alveolar stage is thought to be gonadotropin dependent (Lubzens, 2010), and recent work in coho salmon (*Oncorhynchus kisutch*) has also indicated that progression into the cortical alveolar stage is associated with an increase in E2 (Campbell et al., 2006).

The decrease in T with increasing GSI in “RFs” could indicate a decline associated with final maturation. Final maturation of oocytes in fishes typically occurs with

declining levels of sex hormones (Nagahama, 1994). Kulikova (1985) found that human chorionic gonadotropin (a leutinizing hormone analog) is effective in stimulating oocyte growth in female round gobies (Kulikova, 1985), but when it was delivered in late stages of development it delayed spawning, which could indirectly indicate that a reduction in steroid hormone levels is required for the hormonal milieu during maturation. However, of the two steroid hormones, E2 typically declines more commonly than testosterone (Methven et al., 1992, Kime, 1993, Rinchard et al., 1997). Future studies are needed to examine the role of testosterone in final oocyte maturation.

Males

The 1% GSI cutoff effectively categorized males into those with sperm and those without sperm, but androgen levels showed no correspondence with GSI within both “RMs” and “NRMs”. The results within “NRMs” indicated that cellular and endocrine changes are occurring before gross increases in gonad size. Such an initiation of spermatogenesis prior to increase in GSI has also been observed in the African cichlid *Astatotilapia burtoni* (Maruska et al., 2011). Similarly, although mature sperm were observed in all “RMs”, the degree of active spermatogenesis occurring will be variable and cannot be described with a simple GSI measure. In fact, the GSI may show closer correspondence with the proportion of cells in earlier stages of development, rather than mature sperm. In male Atlantic stingrays (*Dasyatis sabina*), for example, GSI reaches a peak and begins to decline prior to maximum sperm production (Maruska et al., 1996). Researchers should take care in using and interpreting male round goby GSI data because this measure provides limited detail on gonadal and hormonal condition.

Despite the lack of association between hormone level and GSI, 11KT and T likely both play important roles in round goby testis development. The trend for an increase in 11KT level during the transition from immature to developing gonad stages could indicate that 11KT plays a role in early stages of spermatogonial development. 11-ketotestosterone is generally more effective than T in stimulating spermatogenesis in fishes (Miura et al., 1991, Borg, 1994, Cavaco et al., 1998, Schulz, 2010). Additionally, the concurrent elevation of 11KT and T is commonly observed in fishes and typically relates positively with testis development (Modesto and Canario, 2003, Sisneros et al., 2004, Butts et al., 2011).

Seasonality

The presence of spawning-capable fish throughout the summer indicates that round goby spawning events are occurring throughout the summer. There were no clear seasonal patterns in hormone level, which could be because these analyses were limited to spawning-capable fish, which are expected to be in similar hormonal states. The proportion of reproductive female round gobies decreases as the reproductive season progresses, in both North America and Europe (MacInnis and Corkum, 2000, Young et al., 2010). The length of the spawning season is expected to vary with environmental conditions, including temperature (Charlebois et al., 1997).

There was a trend for a decline in female GSI as the season progresses. This could indicate that a cohort of younger fish with lower fecundity are spawning, which would be consistent with MacInnis and Corkum's (2000) finding of a higher proportion of age 1

females in June and July. Alternatively, a decline in GSI with the progression of the season could indicate that female round goby batch size decreases with subsequent batches. For example, in Atlantic cod, another multiple spawning teleost, the number of developing oocytes in females decreases as the season progresses (Kjesbu et al., 1996).

Since reproductively mature fish can be found throughout the breeding season, it is likely that a wide range of estrogen values will be observed throughout the summer. Relatively constant seasonal patterns of E2 levels are often common over the reproductive season in multiple spawners because gonadal recrudescence is occurring during the breeding period (Rinchard et al., 1996, 1997). In Atlantic halibut, E2 fluctuates within each batch, but decreased overall as the season progresses (Kjesbu et al., 1996). Elevated steroid hormone levels during the breeding period in multiple spawning fish contrasts patterns in single spawners, where E2 and T are highest weeks or months before spawning (Scott et al., 1980, Sisneros et al., 2004).

Conclusions

In studies where the reproductive condition of fish is critical, I suggest researchers examine histological stage of gonads, as GSI cutoffs encompass fish in various stages of development. Most adult females captured during the summer can be considered to be vitellogenic, and egg recruitment appears to occur gradually between batches. Due to the relatively continual oocyte growth in between batches and the long breeding season, steroid hormone and GSI are both likely a poor indicator of reproductive season in the round goby. The hormone levels reported in this study lay a groundwork for future

studies in behavioural and sensory reproductive endocrinology of this species, as well as potentially studies on endocrine disruption (Marentette et al., 2010, Bowley et al., 2010).

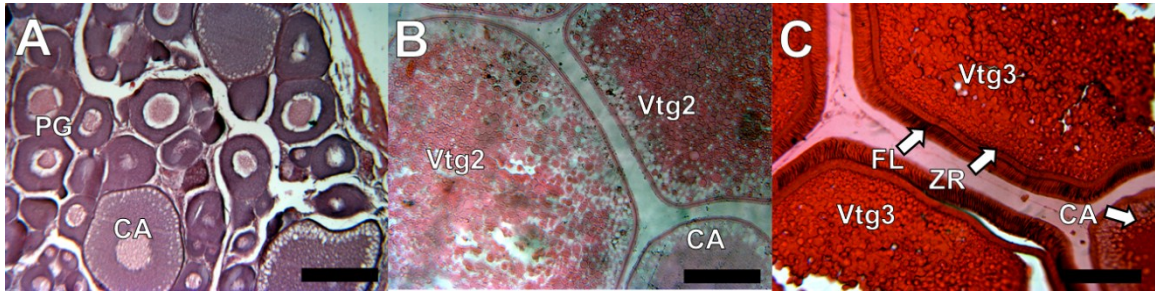
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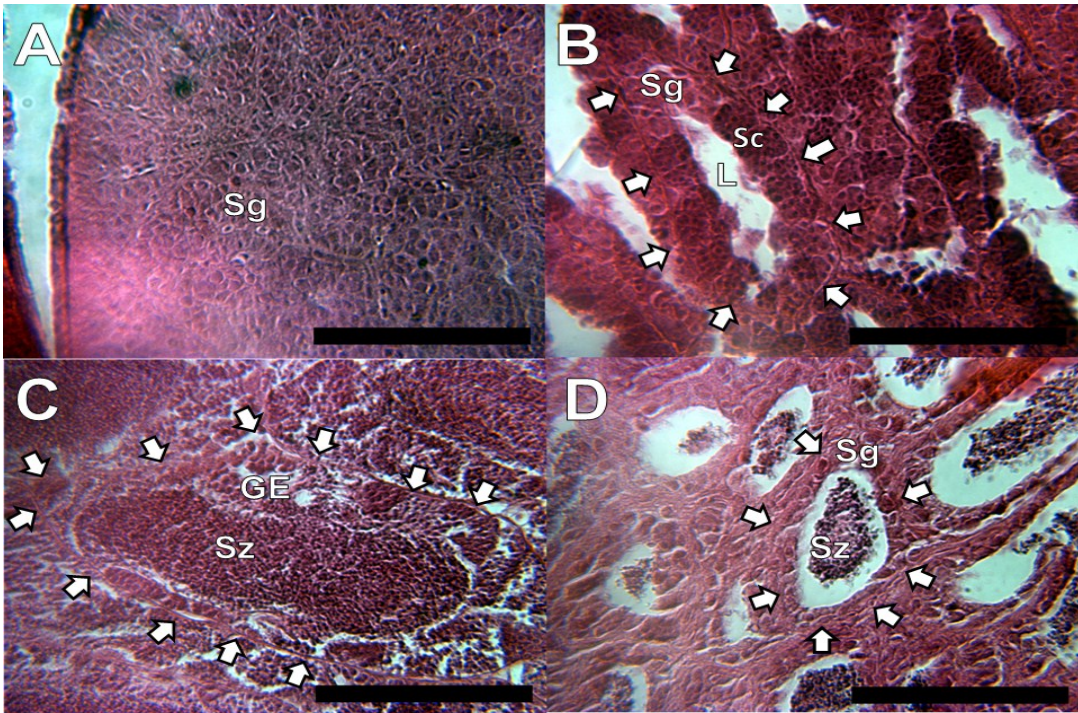
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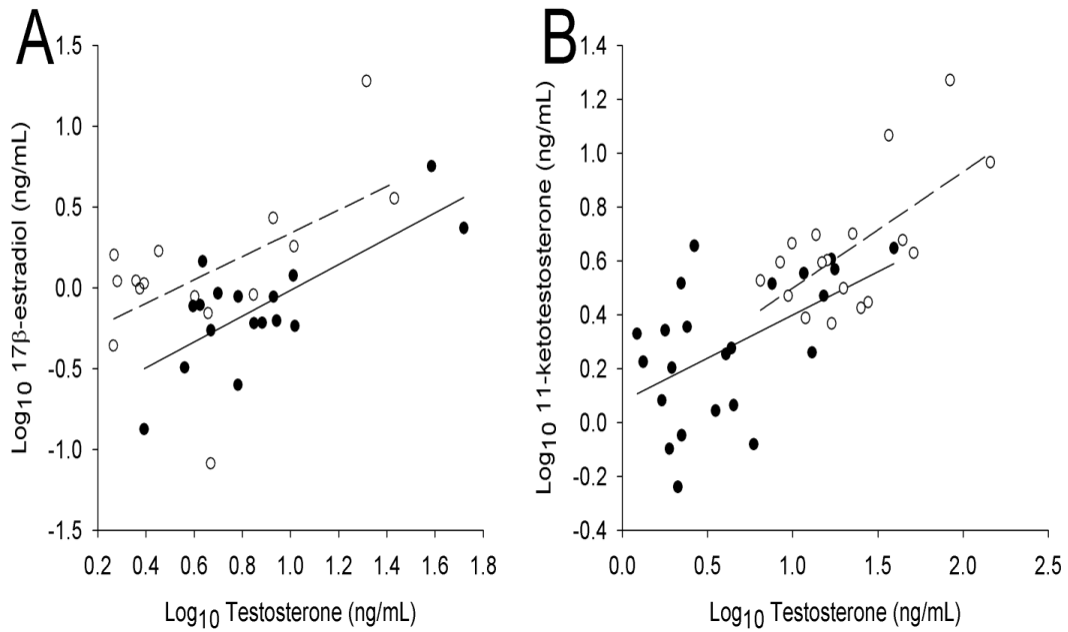
Figures



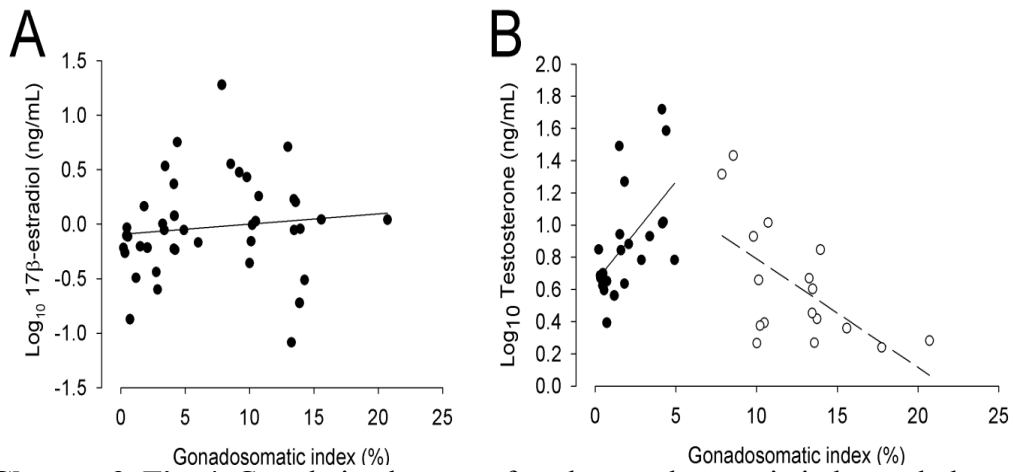
Chapter 3, Fig. 1. Light micrographs of ovaries in cortical alveolar (A), developing (B), and spawning capable (C) stages. PG = primary growth oocyte, CA = cortical alveoli oocyte; Vtg2 = stage 2 vitellogenic oocyte, Vtg3 = stage 3 vitellogenic oocyte; ZR = zona radiata; FL = follicle layer. Scale bars represent 200 μ m.



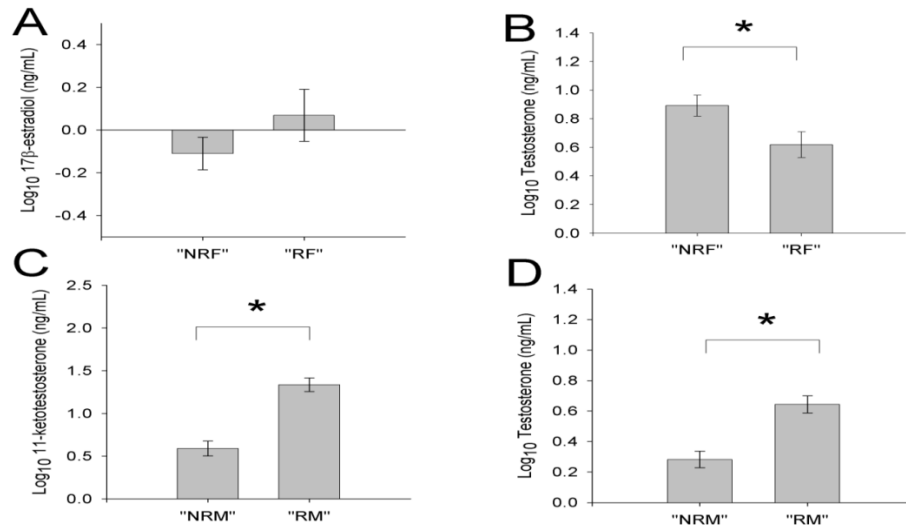
Chapter 3, Fig. 2. Light micrographs of testes in immature (A), developing (B), spawning capable (C), and regressing (D) stages of development. SG = spermatogonia, SC = spermatocytes, SZ = spermatozoa; L = lumen of lobule; GE = germinal epithelium. Seminiferous lobules are outlined with arrows in panels B,C and D. Scale bars represent 100 μ m.



Chapter 3, Fig. 3. (A) The association between testosterone and 17β-estradiol in females. Separate lines are fit for “NRFs” (filled circles, solid line) and “RFs” (open circles, dashed line). (B) The association between testosterone and 11-ketotestosterone in males (“NRMs” = filled circles, solid line; “RMs” = open circles, dashed line). Testosterone values are adjusted based on reproductive group extraction recoveries (Table 1).

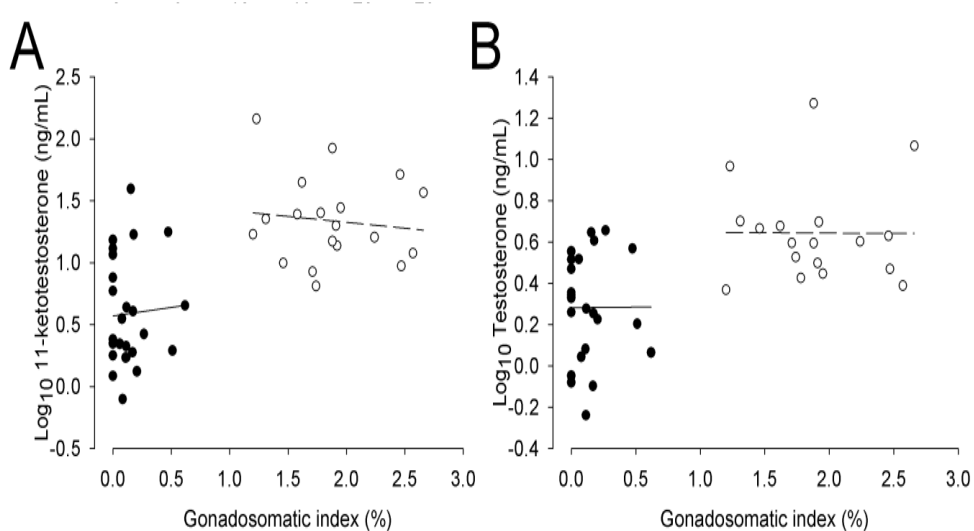


Chapter 3, Fig. 4. Correlation between female gonadosomatic index and plasma 17β-estradiol (A) and testosterone (B). In panel A, a single line represents the overall correlation between GSI and E2, whereas separate correlations were performed for each reproductive group in panel B.

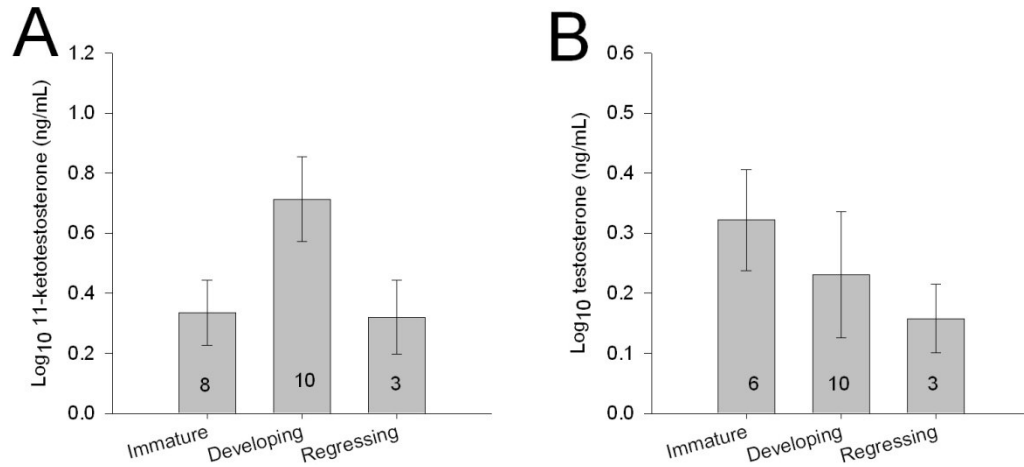


Chapter 3, Fig. 5. Hormone levels in female (A,B) and male (C,D) reproductive groups, as determined by GSI cutoffs (GSI >1 and 8% in males and females, respectively).

Androgens are adjusted to 100% according to pooled extraction efficiencies (Table 1).

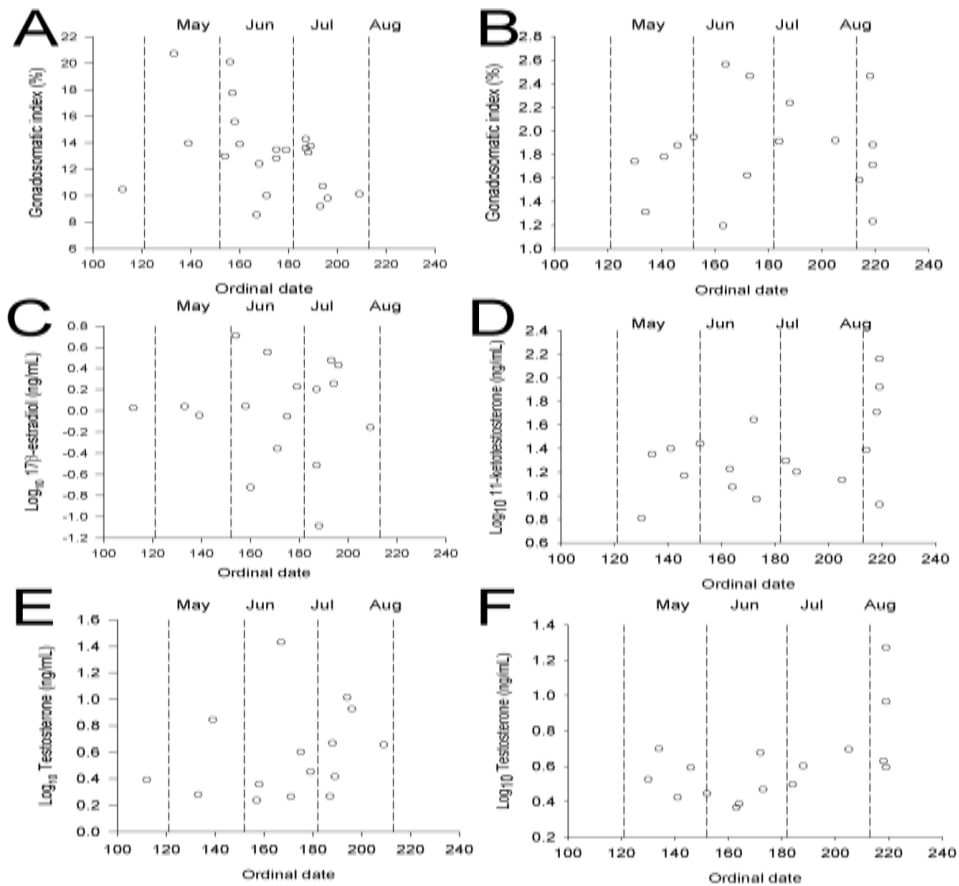


Chapter 3, Fig. 6. Relationship between gonadosomatic index and (A) 11KT and (B) T in males. Separate regressions were performed for “NRMs” (filled circles, solid lines) and “RMs” (open circles, dashed lines) due to differences in androgen extraction recoveries between the groups. Values are adjusted to 100% according to pooled extraction efficiencies (Table 1).



Chapter 3, Fig. 7. (A) 11-ketotestosterone and (B) testosterone levels in “NRMs”

according to histological staging. Regressing males were excluded from statistical tests for both androgens due to small sample size. Sample sizes are indicated within each bar.



Chapter 3, Fig. 8. Trends in gonadosomatic index and reproductive hormones through the summer of 2011. (A,C,E) Scatterplots relating ordinal date to female gonadosomatic index (A), 17 β -estradiol (C), and testosterone (E). (B,D,F) Scatterplots relating ordinal date to “RM” gonadosomatic index (B), 11-ketotestosterone (D), and testosterone (E). Reference lines indicate the first day of each month. Data are restricted to reproductive fish (GSI above 1% and 8% cutoffs in males and females, respectively).

Tables

Chapter 3, Table 1. Summary of extraction recoveries for testosterone (T), 11-ketotestosterone (11KT), and 17 β -estradiol (E₂) tested from pooled plasma from each reproductive group (reproductive males = “RM”, non-reproductive males = “NRM”, reproductive females = “RF”, non-reproductive females = “NRF”). Symbols § and * indicate similar extraction efficiencies between 11KT and T from separate assays.

Hormone	Reproductive group pool	Plasma volume (μ L)	Spike on curve (pg/mL)	Recovery (%)
T	“RM”	7.7	40	13 [§]
T	“NRM”	4.4	40	49*
11-KT	“RM”	1.0	25	18 [§]
11-KT	“NRM”	1.0	25	56*
T	“NRF”	1.0	40	31
T	“RF”	1.0	40	90
E ₂	NRF	4.0	100	115
E ₂	RF	2.1	100	95

Chapter 3, Table 2. Criteria used for classifying reproductive stage using round goby gonads (Adapted from Brown-Peterson et al., 2011).

Sex	Stage	Histological features
Females	Immature	Only oogonia and primary growth oocytes present
	Developing	Cortical alveolar and/or vitellogenic oocytes (Vtg1 and Vtg2) are present. Yolk granules in vitellogenic oocytes are small, not filling the entire cytoplasm
	Spawning capable	Yolk globules densely packed throughout the oocyte (Vtg 3)
Males	Immature	Only spermatogonia present, no lumen developed within lobules
	Developing	Lumen formation, spermatocytes present. Spermatids and spermatozoa may also be present in spermatocysts, but they have not yet started accumulating in the lumen
	Spawning capable	Spermatozoa present in the lumen of the spermatocytes, and some spermatogenesis occurring in germinal epithelium
	Regressing	Residual reserves of spermatozoa present in the lumen or sperm duct

Chapter 3, Table 3. Numbers and GSI (mean \pm s.e.m) of fish categorized into each gonadal stage in relation to GSI groupings (CA stage = cortical alveolar stage).

GSI group	Total	Stage							
		Immature/CA stage		Developing		Spawning capable		Regressing (males)	
		N	GSI	N	GSI	N	GSI	N	GSI
"NRM"	21	8	0.05 \pm 0.02	10	0.2 \pm 0.06	0	-	3	0.3 \pm 0.13
"RM"	16	0	-	0	-	16	1.9 \pm 0.1	0	-
"NRF"	17	3	0.7 \pm 0.3	10	3.0 \pm 0.5	4	3.9 \pm 0.3	-	-
"RF"	21	0	-	0	-	21	13.0 \pm 0.1	-	-

CHAPTER IV: CONCLUSIONS AND RECOMMENDATIONS

This thesis advances our understanding on the physiological conditions which may generate variability in auditory function. Specifically, auditory function may vary with sex, reproductive state, and growth. The results also highlight the importance of examining endocrine condition in sensory physiology and behavioral studies.

In Chapter 2, E2 level was associated with changes in auditory function, despite no differences between “NRFs” and “RFs”. In addition, sex differences in hearing were observed, yet they interacted with growth and E2 level to affect auditory function. Taken together, the results from Chapter 2 indicate that multiple factors of a receiver’s internal physiological state can influence auditory function.

Chapter 3 indicated that while GSI cutoff values are effective in making gross classification of reproductive development, these categories encompass individuals in varied endocrine states. Many studies rely on GSI to determine reproductive state, particularly in females. The GSI assumes that gonad volume and gonad state are related, but this assumption is not always valid. The findings from Chapter 3 should inform experimental design for researchers studying round goby reproductive biology.

Examining fluctuation in hormone levels across the reproductive cycle is a significant step towards further understanding the reproductive biology of the round goby. The finding that E2 was associated with changes in auditory processing contributes to emerging evidence for neuroendocrine coupling of reproductive signaling and sensory reception in the round goby. Meunier et al. (2009) observed that after gravid females were introduced to a nest guarding male in the laboratory, a full 10 days elapsed prior to spawning. This indicates the potential importance of dynamic signalling between males

and females in late stages of courtship, which could be under neuroendocrine control. Social triggers via auditory, olfactory and visual modalities likely stimulate the brain-pituitary-gonadal axis to coordinate mating behaviours. Conspecific acoustic input can trigger leutinizing hormone release in birds (Cheng et al., 1998); the conspecific interactions between male and female round gobies leading up to spawning could similarly initiate gonadotropin release to stimulate ovulation and spermiation. Steroid hormones such as E2 could prime the auditory and olfactory systems during gonadal recrudescence to allow for the encoding of sensory stimuli required for final maturation and spawning.

The central role of gonadal steroids in pheromonal communication in the round goby (Murphy and Stacey, 2002, Belanger et al., 2007) is an additional motivation to proceed with other studies in this area. In round goby pheromonal communication, both the production and reception of olfactory signals are reproductive state dependent (Murphy and Stacey, 2002, Gammon et al., 2005, Belanger et al., 2007). Moreover, E2 and estrone (a possible E2 precursor) are detected by the olfactory system of males (Belanger et al., 2007).

The current thesis contributes to our understanding of the ecological and physiological mechanisms generating condition- and sex-dependent auditory function. Other fish audition studies failing to observe reproductive condition differences in auditory sensitivity have occurred in species which seem to rely on acoustic communication year round (Maruska et al., 2007, Vasconcelos et al., 2011a). Round gobies exhibit seasonal reproduction patterns and seasonal migration to breeding sites, as seen in the plainfin midshipman (Sisneros et al., 2004), which led to the prediction that a

seasonal change in auditory function could be an adaptive trait. However, round gobies have a flexible reproductive strategy and a nearly continuous reproductive capacity if environmental conditions are favourable (Moiseyeva and Rudenko, 1976, Kulikova, 1985). Dynamic hormonal modulation of auditory neural function, potentially via rapid, non-transcriptional mechanisms (Remage-Healey and Bass, 2006), could be a mechanism to facilitate reproductive behaviours in the round goby. Such a control mechanism would resemble the endocrine control of auditory function observed in the Hawaiian sergeant damselfish, *Abudef abdominalis*, which despite exhibiting no overall effect of reproductive season on auditory function, exhibits GnRH-dependent regulation of auditory processing (Maruska et al., 2007, Maruska and Tricas, 2011).

In Chapter 2, the lower thresholds in females at high tone frequencies in conjunction with potential E2 effects on auditory filtering indicate that female encoding of auditory signals likely differs from males. However, there were no sex differences in auditory threshold at 100-200 Hz or in response to the pulse, which could indicate that indicating that auditory sensitivity may not differ between the sexes when receivers are within a critical range from the source (see sound propagation discussion below). Sex differences in auditory function may not always be evident in overall sensitivity, but rather arise in how the auditory information is encoded (e.g. Hoke et al., 2008, 2010, Henry and Lucas, 2010).

Studies in fish acoustic communication should move beyond the simple tone audiogram, since communication occurs above threshold and tone responses do not directly reflect responses to natural stimuli (Maruska and Tricas, 2009). Moreover, aspects of receiver condition (e.g. sex, age/growth, reproductive condition) may affect

hearing in a stimulus specific manner (Miranda and Wilczyński, 2009, Maruska and Tricas, 2011) and may only be evident using certain hearing metrics. For example, in the Carolina chickadee (*Poecile carolinensis*), males have greater auditory sensitivity than females, yet females perform better on measures of frequency discrimination (Henry and Lucas, 2009). Additionally, the frequencies eliciting maximum responses may vary as a function of sound level above threshold (Lu et al., 2004). Thus, an examination of sensitivity should be decoupled from assessments of auditory processing above threshold. Some studies in fish acoustics have used conspecific stimuli in AEP setups, but the techniques used to analyze the data acquired are in early stages (e.g. Wysocki and Ladich, 2003, Belanger et al., 2010, Vasconcelos et al., 2011b). Cross correlation techniques commonly used in human ABR studies to examine signal fidelity and phase locking could be a promising area of investigation in fish acoustics (Skoie and Kraus, 2010).

Propagation studies of round goby vocalizations in the field would allow for a better assessment of the salient features of the acoustic stimulus at distances of several meters (e.g. Mann and Lobel, 1997), which could more fully address whether the auditory threshold of females in the high frequency range is of significance for the active space of male vocalization detection. The range of the particle motion near field is wavelength dependent, with lower frequencies having a greater range (Bass and Clark, 2003). However, the propagation of low frequencies is hampered in shallow waters, and higher frequencies should be favoured for long range acoustic communication in shallow waters due to the cutoff frequency (Bass and Clark, 2003). For example, in a study of

propagation of damselfish sounds in shallow water, frequency bands at 400 and 500 Hz exhibited less attenuation than lower frequencies (Mann and Lobel, 1997).

Propagation can also vary dramatically based on substrate in shallow waters (Bass and Clark, 2003). The rocky substrates favoured by round gobies (Young et al, 2010) should minimize sound absorption and favour a decrease in the cutoff frequency, resulting in greater propagation of vocalizations. Most propagation studies to date measure sound in terms of pressure, but round gobies lack an air bubble or swim bladder to transduce pressure waves to the inner ear. As accelerometer technologies become more commercially available, field studies on particle motion should be fruitful research avenues for examining the active space of round goby vocalizations.

Overall, the current research highlights the dynamic nature of sensory and reproductive physiology. I have elucidated some potential mechanisms through which the auditory system may interact with the reproductive system to influence reproductive behaviours. In general, the findings indicate that the overt behavioural responses of animals are the result of complex interactions between physiological systems within the body.

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APPENDIX

Cold spikes used in extraction recovery, plasma dilutions

Sample ID	Hormone	Standard solution supplied in Cayman kits (ng/mL)	Standard solution used in spikes (pg/mL)	Spike volume (uL)	Final spike amount (on standard curve) (pg/mL)	Volume of plasma assayed (uL)	Volume of assay buffer	Dilution
RM	T	10	600	12.00	40	7.7	180	1:24
RM+spike	T	10	600	12.00	40	7.7	180	1:24
NRM	T	10	600	12.00	40	4.4	180	1:41
NRM+spike	T	10	600	12.00	40	4.4	180	1:41
NRF	T	10	600	12.00	40	1.0	180	1:180
NRF spike	T	10	600	12.00	40	1.0	180	1:180
RF	T	10	600	12.00	40	1.0	180	1:180
RF+spike	T	10	600	12.00	40	1.0	180	1:180
RM	11-KT	50	500	9.75	25	1.0	195	1:195
RM+spike	11-KT	50	500	9.75	25	1.0	195	1:195
NRM	11-KT	50	500	9.75	25	1.0	195	1:195
NRM+spike	11-KT	50	500	9.75	25	1.0	195	1:195
NRF	E2	400	600	30.00	100	4.0	180	1:36
NRF+spike	E2	400	600	30.00	100	4.0	180	1:36
RF	E2	400	600	30.00	100	2.1	180	1:87
RF+spike	E2	400	600	30.00	100	2.1	180	1:87

Raw data for pooled extraction recoveries

Sample ID	Hormone	[Well 1] (pg/mL)	[Well 2] (pg/mL)	[Well 3] (pg/mL)	[Mean wells] (pg/mL)	Extraction efficiency (%)	
RM	T	90.7	92.3		91.5		
RM+spike	T	98.0	95.7		96.9	13.4	←RM
NRM	T		52.3	54.7	53.5		
NRM+spike	T	72.5	74.1		73.3	49.3	←NRM
NRF	T	77.4	78.7		78.0		
NRF spike	T	83.7	97.5		90.6	31.4	←NRF
RF	T	26.0	34.5	45.5	35.4		
RF+spike	T	65.5	77.4		71.4	90.2	←RF
RM	11-KT	41.2	40.6	42.6	41.5		
RM+spike	11-KT	45.5		46.2	45.9	17.7	←RM
NRM	11-KT	9.6	9.9	9.7	9.7		
NRM+spike	11-KT	23.7		23.6	23.7	55.7	←NRM
NRF	E2		122.2	134.4	128.3		
NRF+spike	E2	242.8	244.3		243.6	115.2	←NRF
RF	E2	30.4	32.0		31.2		
RF+spike	E2		129.8	123.4	126.6	95.4	←RF

Equations used to calculate spikes for recoveries:

1. $\text{Concentration of diluted spike solution} * \left(\frac{\text{Spike volume (uL)}}{\text{Volume of assay buffer (uL)}} \right) =$
 $\text{Concentration of spike on the curve}$
2. $\text{Concentration of stock standard} * \left(\frac{\text{Volume of stock standard}}{\text{Total volume of diluted spike solution desired}} \right) =$
 $\text{Concentration of diluted spike solution}$

Ether Extraction Protocol

1. Label one test tube (16x100 mm) and one scintillation vial for each sample
2. Pipette out plasma sample into test tube and add 1 mL of double-distilled water. Vortex.
3. Add 4 mL of ether to each test tube using a plastic pipetter. Vortex for 20 seconds until central whirlpool reaches the bottom of the test tube, increasing speed slowly from 1-6. Do this for each tube in sequences. Repeat 3 times, allowing the ether layer to settle each time.
4. Cover test tubes with paraffin and place test tube rack in -80°C for 17 min to freeze the aqueous phase. Transfer test tube rack in ice to -20C and retrieve each test tube individually to carefully decant the ether phase into the scintillation vial.
5. Allow ether to evaporate overnight in fume hood.
6. Prior to assay, reconstitute steroids in EIA buffer, vortexing bottom and sides of scintillation vial several times to level where ether reached the day before.

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