

## RESEARCH ARTICLE

# Condition-dependent auditory processing in the round goby (*Neogobius melanostomus*): links to sex, reproductive condition and female estrogen levels

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### SUMMARY

Neural responses to sensory stimuli often differ between sexes, vary seasonally, and can be regulated by endocrine activity, but the ecological and physiological mechanisms driving such patterns are not well understood. The current study examined how auditory function in the round goby (*Neogobius melanostomus*), a vocal teleost, co-varied with sex, reproductive condition and female plasma  $17\beta$ -estradiol level. Auditory evoked potentials were collected in response to tone pips (100–600 Hz) and a natural round goby pulse vocalization. Additionally, saccule hair cell densities were compared across reproductive groups. Auditory threshold was evaluated in terms of pressure and particle acceleration, and response amplitude and onset latency were measured at 10 dB above threshold. Relative to males, females displayed lower auditory thresholds in response to the natural vocalization and to tones at 300–600 Hz, and had a higher density of saccule hair cells. The  $17\beta$ -estradiol level was positively associated with amplitude and latency for the pulse stimulus and with both threshold and amplitude for tones at 100–200 Hz in females. Relative to non-reproductive males, reproductive males exhibited longer response latencies at 100–200 Hz. The results demonstrate sexual dimorphism in auditory function in a teleost fish as well as intra-sexual variation, partially based on hormone levels. The current research further identifies links between auditory function and reproductive behaviors in fishes and provides a finer-scaled analysis of how this behavior is reflected at the level of the sensory systems facilitating signal reception.

Key words:  $17\beta$ -estradiol, acoustic communication, sexual dimorphism, reproductive plasticity, hair cells.

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### 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, with production and response traits likely coupled evolutionarily (Endler, 1992). Sender and receiver traits are also likely coupled on a temporal scale within an organism's lifetime; when there is seasonal variation in signal production, for example, there may also be potential for seasonal plasticity in auditory function. Seasonal and reproductive state-dependent changes in auditory function could serve an adaptive function by minimizing costs associated with maintaining sensory neurons (Niven and Laughlin, 2008). In addition to such condition-dependent effects on audition, males and females typically attend to conspecific acoustic signals with different purpose (e.g. agonistic versus courtship), which could be reflected by sex differences in auditory function (Wilczynski et al., 1984; Searcy and Brenowitz, 1988; Gall et al., 2011). Taken together, these observations indicate that multiple aspects of receiver condition may influence auditory function to promote reproductive behaviors.

Many fishes show annual patterns of reproductive activity and rely on acoustic communication for mating behaviors (Zelick et al., 1999; Bass and McKibben, 2003; Kasumyan, 2009). Neuroendocrine studies in acoustic and weakly electric fishes indicate that endocrine regulation of signal production and reception could be a widespread control mechanism for acoustic behaviors in fishes and in other vertebrate groups (Bass and Zakon, 2005). For

example, in plainfin midshipman, *Porichthys notatus*, implantation of non-reproductive females with estrogen and testosterone results in increased phase-locking (matching neural spike rate to stimulus cycle) precision of harmonics in 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, gonadotropin-releasing hormone (GnRH) modulates auditory processing during the reproductive period in the banded damselfish, *Abudefduf abdominalis* (Maruska and Tricas, 2011), and in the African cichlid *Astatotilapia burtoni*, changes in auditory function are associated with sex steroid fluctuations and reproductive stage (Maruska et al., 2012). Acoustic communication in teleost fishes is therefore a compelling study system for examining the neuroendocrine control of reproduction.

In the current study, we examined condition-dependent auditory function in the round goby, *Neogobius melanostomus* (Pallas 1814), a vocal benthic teleost. Several aspects of the round goby's breeding biology indicate that this species may provide a useful model system for examining 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 ~180 Hz) during occupancy (Rollo et al., 2007), which may serve to facilitate female attraction and/or deter intruders (Rollo et al., 2007; Rollo and Higgs, 2008; Kasurak et al., 2012). Vocal behaviors have only been observed in male round gobies during the breeding

season (Rollo et al., 2007), similar to what has been shown for other members of the Gobiidae (Tavolga, 1956; Lugli et al., 1997), indicating that acoustic communication is seasonally dynamic and linked to reproduction. Females arrive on the breeding grounds after males (Kotvun, 1980) and may retreat to deeper waters between spawning batches to avoid predation (Young et al., 2010). In playback experiments, females show a stronger propensity to approach acoustic stimuli than males (Rollo et al., 2007), which could indicate a female advantage in auditory function to aid in localizing vocalizing males. Additionally, female attraction to male olfactory–auditory signals is stronger in gravid females than in non-gravid females (Kasurak et al., 2012), and female olfactory sensitivity and attraction to male odors is enhanced in gravid females (Belanger et al., 2004; Gammon et al., 2005).

Using auditory evoked potential audiometry (AEP), we examined the effects of sex and reproductive condition on hearing ability in the round goby. In addition, we related female hearing measurements to plasma 17 $\beta$ -estradiol (E2) – a crucial hormone for reproductive development in female fishes (Kime, 1993) – and tested whether variation in hearing ability in both sexes was associated with hair cell density in the saccule, the primary auditory end organ in fishes (Popper and Fay, 1999) [but see Lu et al. for roles of utricle and lagena (Lu et al., 2003; Lu et al., 2004)]. Auditory threshold, suprathreshold response amplitude and suprathreshold latency were measured in response to tones and a single-pulse round goby vocalization. We predicted that females would have superior hearing ability relative to males (lower thresholds, higher response amplitudes, shorter latencies), and that plasma E2 level would correlate positively with enhanced auditory phenotypes in females. No specific predictions were made about the effect of reproductive condition on hearing ability in males because while heightened sensitivity to neighboring male vocalizations could be advantageous for assessment purposes, it could also conceivably increase the probability of engaging in costly agonistic behaviors.

## MATERIALS AND METHODS

### Subjects

Fish were collected by hook and line from the Detroit River shoreline in Windsor, ON, Canada (42°18'23.83"N, 83°4'32.46"W) between May and August 2010 and 2011. Reproductive fish were tested within 5 days of capture except for two females, which became reproductive in the laboratory (details on reproductive condition criteria are given below). Non-reproductive fish were brought into the lab and tested at various dates over the course of the summer. Fish kept in the laboratory were fed fish flakes (Tetramin Inc., Blacksburg, VA, USA) and housed with conspecifics in mixed-sex communal tanks on a 16h:8h light:dark photoperiod. Body mass and total length (TL) (means  $\pm$  s.e.m.) of fish used in the study were as follows: non-reproductive females (NRF): mass 5.96 $\pm$ 0.38 g, TL 8.07 $\pm$ 0.19 cm; reproductive females (RF): mass 6.81 $\pm$ 0.47 g, TL 8.1 $\pm$ 0.16 cm; non-reproductive males (NRM): mass 16.16 $\pm$ 3.09 g, TL 10.81 $\pm$ 0.51 cm; reproductive males (RM): mass 23.11 $\pm$ 4.55 g, TL 12.70 $\pm$ 0.93 cm. Details on sample sizes are given in the following sections. All work was conducted under University of Windsor approved Animal Care Committee permits under Canadian Council for Animal Care (CCAC) guidelines.

### Assessment of reproductive condition

Following hearing tests, fish were killed by clove oil overdose and reproductive condition was assessed using a gonadosomatic index (GSI):  $GSI = \text{total gonad mass} / \text{total body mass} \times 100$  (Crim and Glebe, 1990), with mass measured to the nearest 0.01 g (Scout Pro,

SP202, Ohaus Corp., Pine Brook, NJ, USA). Reproductive condition assessment involved an initial sorting as 'reproductive' if males possessed the characteristic dark reproductive coloration and swollen cheeks (Marentette et al., 2009) and if females possessed swollen abdomens (Kasurak et al., 2012). The initial sorting was verified after euthanasia using conventional GSI cut-off values in round goby literature. 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). GSI within each reproductive group was as follows: NRF 1.5 $\pm$ 0.35%, RF 13.2 $\pm$ 0.94%, NRM 0.17 $\pm$ 0.005%, RM 1.6 $\pm$ 0.12% (means  $\pm$  s.e.m.). In addition to GSI measurement, selected females 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 a paper towel (without anesthesia because of the non-invasive nature of the AEP technique) on a platform stationed 0.76 m from the speaker at a water depth of 10–12 cm. The paper towel was pinned to the clay bedding of the platform and also clipped to the vertical platform arm. Fish were able to breathe normally, but could not otherwise move their head or body throughout the experiments. Sound levels of all stimuli presented were calibrated at the location of the fish's head before each test day using the maximum cycle root mean square (r.m.s.) value output from an oscilloscope (TDS1002, Tektronix Inc., Beaverton, OR, USA) connected to a hydrophone (calibration sensitivity  $-208.9$  dB re. 1 V  $\mu$ Pa $^{-1}$ , Reson Inc., Slangerup, Denmark).

While pure tone stimuli are useful for evaluating variation in relative sensitivity across frequency bandwidths, responses to complex, natural stimuli may stimulate the auditory system differently from pure tones as a result of differences in total energy (e.g. Maruska and Tricas, 2009; Belanger et al., 2010). Additionally, relationships between hearing ability and sex or endocrine status can show stimulus specificity (e.g. Miranda and Wilczynski, 2009; Maruska and Tricas, 2011). Thus, in addition to tones, all fish were presented with a single 96.7 ms pulse male vocalization (Fig. 1A,B). The stimulus was extracted from a geophone recording of a pulse train produced by 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 s $^{-1}$ . Tone pip stimuli consisting of 10 ms sine waves gated with 2 ms Hanning windows were presented at 100, 200, 300, 400, 500 and 600 Hz at a rate of 8 s $^{-1}$ . The tone frequency range was chosen based on a previous study which indicated that acoustic detection in round goby does not commonly occur above approximately 600 Hz (Belanger et al., 2010). 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 (see 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 deg and 270 deg, 1000 presentations in each phase) with sets of responses from opposing phases averaged to reduce stimulus artifact. Stimuli

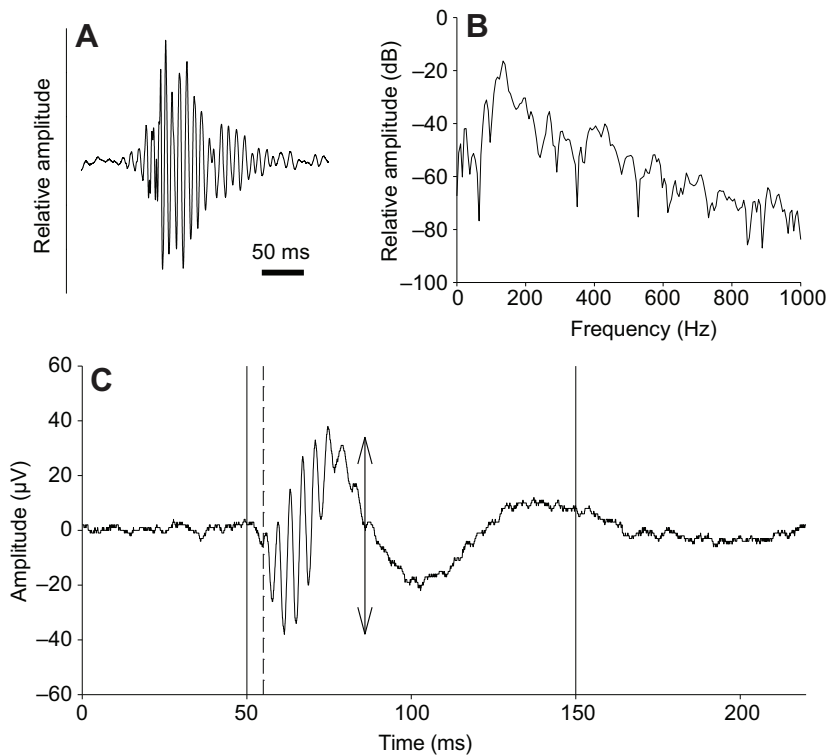


Fig. 1. (A) Waveform of the male round goby pulse stimulus, as recorded from a hydrophone positioned at the location of the recording platform in the experimental tank. (B) Fast Fourier transform (8192 point Hanning window) generated from 9 s of tank-recorded pulse repetition. (C) A representative auditory evoked potential (AEP) response elicited from the pulse stimulus. The response latency is indicated with a dashed line, and peak-to-peak (p-p) amplitude measurement is indicated with a double-headed arrow. Solid lines at 50 and 150 ms indicate the section of the response used for root mean square (r.m.s.) amplitude measurement.

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.

Because round gobies lack a swim bladder and other accessory hearing structures, particle acceleration is the relevant auditory stimulus, which was measured inside the testing tank for all stimuli presented using a triaxial accelerometer (Model 4524,  $10 \text{ mV ms}^{-2}$ , Akoustik Engineering Ltd, Windsor, ON, Canada) connected to a conditioning amplifier (Deltatron conditioning amp, model no. 2693-A-051, Brüel & Kjær Inc., Norcross, GA, USA). Accelerometer readings were taken while the device rested on the testing platform, in an attempt to mimic stimulation as it would occur in the ears of fish secured to the recording platform. These recordings were taken in another laboratory in an identical tank because of equipment limitations. Acceleration in  $x$ ,  $y$  and  $z$  directions was combined into a single measure ( $A$ ) using the following equation:

$$A = \sqrt{x^2 + y^2 + z^2} \quad (1)$$

Acceleration values were plotted against pressure output from the speaker, and extrapolations from the linear portion of the curve (all  $R^2$  values were greater than 0.99) were used to obtain acceleration estimates at sound levels below the noise floor.

#### Hearing measurements

Auditory threshold, suprathreshold amplitude and suprathreshold latency were measured offline (Fig. 1C). Threshold was assessed visually as the lowest sound level exhibiting an 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 (p-p) amplitude was measured as the difference between the maximum and minimum voltage across the entire recording window, and latency was measured as the time to the trough of the first negative peak of the AEP waveform (Higgs et al., 2003). Additionally, r.m.s. amplitude was calculated for suprathreshold pulse responses to measure the summed neural response over time (Skoe and Kraus, 2010). The r.m.s. amplitude was calculated over the 50–150 ms section of the response (Fig. 1C) after performing a 40 Hz high-pass filter with 20 dB roll-off per octave. Sample sizes in hearing analyses varied slightly depending on audiometric measurement and stimulus (tones *versus* pulse). Despite these differences, the sample sizes in each group were relatively constant across all analyses.

#### Plasma E2 assays

After hearing tests, a subset of females were anesthetized 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 r.p.m. for 10 min (Micro-Hematocrit Centrifuge, LWS-M24, LW Scientific, Lawrenceville, GA, USA) and stored at  $-80^\circ\text{C}$  to be assayed at a later date. Total plasma volumes for fish ranged from 5 to  $40 \mu\text{l}$ . Prior to assay, steroids were extracted once with diethyl ether and assayed in triplicate using ELISAs (Cayman Chemical, Ann Arbor, MI, USA). Plasma dilutions for assays were 1:45 and 1:90 for NRFs and RFs, respectively. Limited plasma volumes precluded the running of extraction efficiencies on all individuals; therefore, extraction recoveries were determined separately for reproductive and non-reproductive females by cold spike recoveries on plasma pools composed of equal volumes from at least 10 individuals from each reproductive group (Bowley et al., 2010). Percentage recovery was  $115.2\%$  for NRFs and  $95.4\%$  for RFs. Inter- and intra-assay variability were  $11.09\%$  and  $11.14 \pm 13.15\%$  (means  $\pm$  s.d.). Plasma levels were  $0.97 \pm 0.36$  in NRFs and  $3.31 \pm 2.25$  in RFs (means  $\pm$  s.e.m.). The sample sizes for analyses involving E2 were 16 for tone analyses and 14 for pulse analyses. Males were



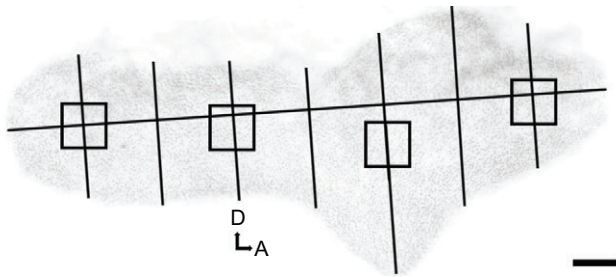


Fig. 2. Phalloidin-labeled saccular hair cell bundles with overlaid line transects and 10,000  $\mu\text{m}^2$  counting boxes (see Materials and methods for details). D, dorsal; A, anterior. Scale bar represents 100  $\mu\text{m}$ .

assayed for 11-ketotestosterone and testosterone, but low extraction recoveries precluded a clear analysis so they were removed from the study.

### Sacculle microscopy

Sacculle epithelia were collected from seven NRF, eight RF, eight NRM and 10 RM in total. Heads were fixed in 4% paraformaldehyde for up to 16 weeks prior to dissection to remove epithelia. Tissues were soaked for 20 min in a 1:16 dilution (stain:phosphate buffer) (Higgs et al., 2002) of Oregon Green phalloidin (Molecular Probes Inc., Eugene, OR, USA), a stain that binds to F-actin fibers in stereocilia bundles (Flock et al., 1982). Multiple micrograph images were captured across the entire epithelium area at  $\times 200$  using a fluorescent microscope (Leica DMIRB inverted fluorescent microscope, Wetzlar, Germany) and stereocilia bundles were manually counted from four 10,000  $\mu\text{m}^2$  boxes distributed across the middle region of each sacculle epithelium using ImageJ (v. 1.44, <http://imagej.nih.gov/ij/>). 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 perpendicular lines, beginning with the line on the most posterior end (Fig. 2). Peripheral regions were excluded from the analysis because of staining and mounting difficulties (tissue tearing and tissue overlapping), which occurred more commonly in these regions.

### Statistical analyses

Analyses testing the 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 analyzed separately. Similarly, tone and pulse thresholds were not compared and were included in separate analyses because these stimuli possess different r.m.s. power spectra. Reproductive group differences for pulses and tone responses were tested with *t*-tests and two-way repeated measures ANOVA, respectively. Tone response repeated measures ANOVA included stimulus frequency as the within-subjects factor and either sex or reproductive condition as the between-subject factor. Following significant ANOVA, *t*-tests comparing averaged responses within low (100–200 Hz) and high (300–600 Hz) frequency categories were performed as *post hoc* tests. Sequential Bonferroni alpha corrections were applied because of the repeated tests on tone responses (two comparisons). The frequency categories characterize a distinction between low frequencies corresponding

to the dominant energy in round goby vocalizations (Rollo et al., 2007) and higher frequencies that will propagate further in shallow waters as a result of 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, E2 was related to averaged responses grouped into the low (100–200 Hz) and high (300–600 Hz) frequency categories described above. Differences in hair cell density among epithelial microregions were tested with a one-way ANOVA. As no effect of location on hair cell density was observed ( $F_{3,95}=2.20$ ,  $P=0.09$ ), data were averaged across boxes. Effects of reproductive condition and sex on hair cell density were assessed using *t*-tests.

Mass and total length were tested as covariates within each sex in all audiometry analyses. Mass and length were not included as covariates in models testing for sex differences because of the low overlap in body size between males and females. Covariates were removed from models when main effects or interaction terms effects had  $P>0.1$  (Engqvist, 2005). Mass and length contributed significantly to models involving male tone amplitude and male pulse threshold and were excluded from all other analyses.

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 because of a lack of sufficient variation) but data fell closely onto the probability plot lines. Plasma E2, body length, tone amplitude, r.m.s. amplitude and particle motion tone threshold were log transformed to meet normality assumptions for hearing analyses. All statistical tests were performed in SPSS (IBM SPSS Statistics, v. 19.0). All descriptive statistics are reported as means  $\pm$  s.e.m.

## RESULTS

### Auditory threshold

Thresholds to the pulse stimulus ( $120.9 \pm 0.068$  dB re. 1  $\mu\text{Pa}$ ,  $2.8 \pm 0.21 \times 10^{-3} \text{ m s}^{-2}$ ) were lower than those to most tones but similar to responses at 100 Hz (Fig. 3). Pressure and particle acceleration thresholds generated similar audiogram profiles; thresholds varied as a function of frequency (pressure:  $F_{5,275}=233.57$ ,  $P<0.001$ , acceleration:  $F_{5,275}=46.01$ ,  $P<0.001$ ), with lowest thresholds at 100 Hz and highest thresholds in mid-range 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 than males in response to the pulse stimulus (mean difference 3.6 dB,  $9.4 \times 10^{-4} \text{ m s}^{-2}$ ) (Fig. 3, Table 1). Similarly, female tone thresholds were lower than those of males, for both pressure and particle acceleration audiograms, and this sex difference was frequency dependent (pressure: sex  $\times$  frequency,  $F_{1,270}=2.03$ ,  $P=0.075$ ; acceleration: sex  $\times$  frequency,  $F_{1,270}=5.41$ ,  $P=0.024$ ). *Post hoc* tests revealed that the sex effect was restricted to the 300–600 Hz category (Table 1); no significant sex differences were found at 100–200 Hz. On average, female thresholds were 2.6 dB ( $5.6 \times 10^{-2} \text{ m s}^{-2}$ ) lower than male thresholds at 300–600 Hz.

Within females, reproductive state based on GSI measurements had no effect on pulse thresholds or tone thresholds (Table 1). However, auditory thresholds were positively associated with E2

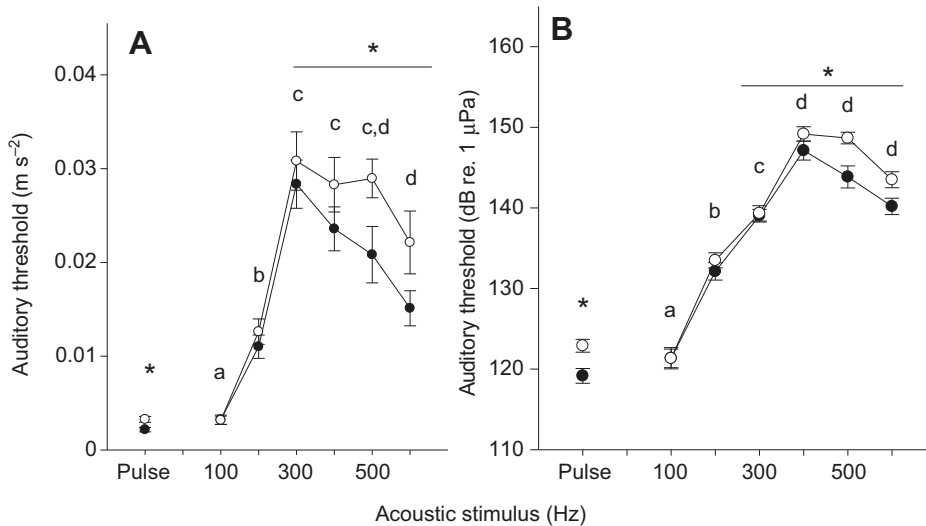


Fig. 3. Auditory thresholds in males (open circles) and females (filled circles) in response to the pulse and tones, expressed in terms of pressure (A) and particle acceleration (B). Sex differences in auditory threshold are indicated by asterisks above stimulus categories (see Materials and methods). 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 the effect of frequency on auditory threshold. Frequencies possessing different letters are significantly different. Error bars are means  $\pm$  s.e.m. in all hearing measurement figures.

at 100–200 Hz (Fig. 4A, Table 1), but not in response to the pulse. Within males, tests of the effects of reproductive condition on pulse threshold showed significant reproductive condition  $\times$  body size interactions (mass:  $F_{1,23}=4.72$ ,  $P=0.04$ ; TL:  $F_{1,23}=4.87$ ,  $P=0.042$ ), which 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 ( $t_{25}=1.39$ ,  $P=0.18$ ). There were no differences between NRMs and RMs in tone threshold (Table 1).

#### Response amplitude

Pulse response amplitudes ( $50.97 \pm 2.77 \mu\text{V}$ ) were greater than amplitudes for most tonal frequencies but similar to responses at 100 Hz (Fig. 5). Tone response amplitude varied as a function of frequency ( $F_{5,245}=14.13$ ,  $P<0.001$ ), with highest amplitudes at 100 Hz (mean  $49.2 \mu\text{V}$ ) and lowest amplitudes at 300 Hz (mean  $28.5 \mu\text{V}$ ).

There was a frequency-dependent effect of sex on tone amplitude (sex:  $F_{1,45}=10.16$ ,  $P=0.003$ ; sex  $\times$  frequency:  $F_{1,45}=2.38$ ,  $P=0.04$ ) (Fig. 5), and *post hoc* tests indicated that female amplitudes were higher than male amplitudes at 300–600 Hz, but not at 100–200 Hz (Table 2). There was no sex difference in pulse amplitude. Female E2 level was positively related to pulse amplitude (Fig. 4B, Table 2).

There was a trend for a positive association between E2 and tone amplitude at 100–200 Hz (Fig. 4C), but not at 300–600 Hz. Female reproductive condition based on GSI had no effect on pulse amplitude or tone amplitude. Within males, there were no differences in pulse amplitude between RM and NRM ( $F_{1,22}=1.80$ ,  $P=0.19$ ). Similarly, after adjusting for significant effects of mass and length on tone amplitude ANCOVA (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:  $F_{1,22}=2.76$ ,  $P=0.11$ ).

#### Response latency

Pulse latencies averaged  $49.4 \pm 0.23$  ms, which was much longer than responses to tones (3.2–17.6 ms), presumably 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. 6). Female E2 level was positively related to pulse latency (Fig. 4D). This hormonal effect was specific to the pulse stimulus, as E2 was not related to latency at 100–200 Hz or 300–600 Hz (Table 2). Reproductive condition had no effect on male pulse latency, but RMs had longer latencies than NRMs in response to tones ( $F_{1,23}=3.84$ ,  $P=0.04$ ). *Post hoc* tests revealed that NRMs

Table 1. Summary of effects on AEP auditory thresholds

Acoustic stimuli	No. fish tested	Receiver conditions			
		Sex	Male reproductive state	Female reproductive state	Female E2
Pulse	( $N=14$ NRF, 13 RF, 16 NRM, 11 RM)	F<M $t_{52}=2.87$ , $P=0.006$	Body size $\times$ condition* Mass: $F_{1,23}=4.72$ , $P=0.04$ TL: $F_{1,23}=4.87$ , $P=0.042$	$F_{1,25}=1.47$ , $P=0.15$	$F_{1,12}=0.46$ , $P=0.51$
100–200 Hz					
Particle acceleration	( $N=13$ NRF, 13 RF, 16 NRM, 14 RM)	$t_{54}=0.7$ , $P=0.49$	n.s. omnibus $F_{1,28}=0.96$ , $P=0.34$	n.s. omnibus $F_{1,24}=0.37$ , $P=0.55$	+ $F_{1,14}=6.06$ , $P=0.027$ , $R^2=0.55$
Pressure	( $N=13$ NRF, 13 RF, 16 NRM, 14 RM)	$t_{54}=0.53$ , $P=0.60$	n.s. omnibus $F_{1,28}=1.42$ , $P=0.24$	n.s. omnibus $F_{1,24}=0.10$ , $P=0.76$	$F_{1,14}=3.53$ , $P=0.081$ , $R^2=0.45$
300–600 Hz					
Particle acceleration	( $N=13$ NRF, 13 RF, 16 NRM, 14 RM)	F<M $t_{54}=2.27$ , $P=0.027$	n.s. omnibus $F_{1,28}=0.96$ , $P=0.34$	n.s. omnibus $F_{1,24}=0.37$ , $P=0.55$	$F_{1,14}=0.28$ , $P=0.61$
Pressure	( $N=13$ NRF, 13 RF, 16 NRM, 14 RM)	F<M $t_{54}=2.92$ , $P=0.005$	n.s. omnibus $F_{1,28}=1.42$ , $P=0.24$	n.s. omnibus $F_{1,24}=0.10$ , $P=0.76$	$F_{1,14}=0.23$ , $P=0.64$

AEP, auditory evoked potential. \*ANCOVA including body mass or total length as a covariate. The + symbol indicates a positive relationship. F, female; M, male; RF, reproductive female; NRF, non-reproductive female; RM, reproductive male; NRM, non-reproductive male; TL, total length.

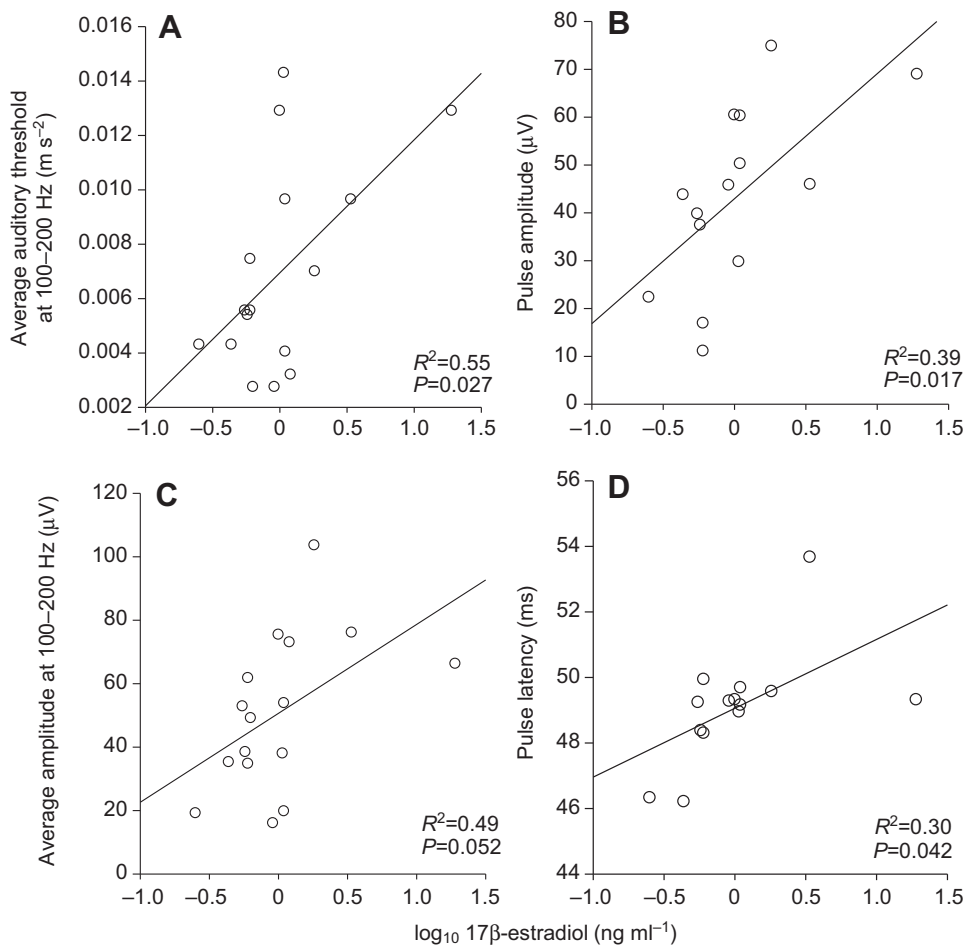


Fig. 4. Linear regressions of female 17 $\beta$ -estradiol (E2) level on (A) average particle acceleration threshold at 100–200 Hz, (B) p–p amplitude in response to the pulse stimulus, (C) average amplitude at 100 and 200 Hz, and (D) latency in response to the pulse stimulus. Each data point in A and C represents an average between subject responses at 100 and 200 Hz (see Materials and methods for details).

had shorter latencies than RMs at 100–200 Hz, but not at 300–600 Hz (Table 2). There were no effects of sex or female reproductive condition on latency, either for the pulse or for tones (Table 2).

#### Sacculle hair cell density

Hair cell density did not differ significantly across counting regions ( $F_{3,95}=2.20$ ,  $P=0.09$ ), so results were collapsed across microregions to generate a single density measurement for each individual. Females had a higher density of hair cells than males, averaging 13.5 more cells per 10,000  $\mu\text{m}^2$  box ( $t_{31}=2.43$ ,  $P=0.021$ ) (Fig. 7). 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

### Female reproductive condition

The prediction that reproductive females would exhibit enhanced auditory function relative to non-reproductive fish was supported by the positive associations between circulating E2 level and pulse amplitude and latency. This finding is consistent with the recent discovery that gravid females display stronger behavioral responses to olfactory–auditory conspecific males signals than non-reproductive females (Kasurak et al., 2012) and highlights a potential link between auditory function and reproductive behavior in female round goby. While the relationships between E2 and hearing measurements are associative rather than due to an experimental manipulation, physiological actions of steroids on the response properties of neurons are well known (Zakon, 1998) and there is

increasing evidence for a stimulatory role of E2 in auditory function (Sisneros et al., 2004; Miranda and Wilczyński, 2009; Tremere et al., 2009; Maruska et al., 2012). E2 could affect the number and kinetics of hair cell ion channels (Zakon, 1998), which could influence the frequency filtering properties of the auditory system, as activation and deactivation rates of ion channels are the main determinants of hair cell resonance (Fettiplace and Fuchs, 1999). More generally, links between sex steroids and sensory processing may be taxonomically widespread because of the close associations between sex steroid fluctuation, gonadal recrudescence, conspecific signaling and mating behavior in many vertebrates (Crews, 1984; Nelson et al., 1990; Munakata and Kobayashi, 2010).

Our finding that female hearing varied with E2 status but not GSI may indicate that female auditory function in round gobies varies on a fine scale across the reproductive cycle, rather than reaching a fixed phenotype at a certain reproductive stage. Fine-scale variation in auditory function has been observed in aseasonal tropical breeders, the Hawaiian sergeant damselfish (Maruska and Tricas, 2009) and the African cichlid *A. burtoni* (Maruska and Tricas, 2011), suggesting that reproductive condition-dependent auditory function may not be limited to species with a limited acoustic season. These findings contrast with previous hypotheses based on work in Batrachoididae, where reproductive condition-dependent auditory function has been related to acoustic seasonality. For example, in plainfin midshipman, acoustic communication appears to be strongly seasonal (Sisneros et al., 2004) and both male and female plainfin midshipman show

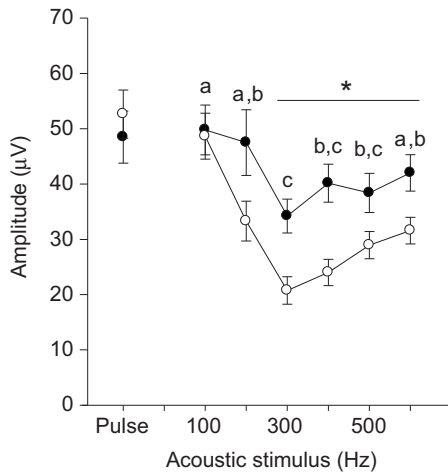


Fig. 5. (A) The p–p amplitudes of AEPs in response to the pulse and tonal stimuli, grouped by sex (males, open circles; females, filled circles). Sex differences are indicated by asterisks above frequency categories. Letters above tone responses indicate results of Tukey HSD *post hoc* tests ( $\alpha=0.05$ ) following a species-wide (males and females combined) test of the effect of frequency on response amplitude. Frequencies possessing different letters are significantly different.

enhanced auditory phenotypes during the reproductive season (Rohmann and Bass, 2011). In contrast, the related Lusitanian toadfish *Halobatrachus didactylus* shows no seasonal changes in auditory function (Vasconcelos et al., 2011a), despite the auditory system being well equipped for conspecific communication (Vasconcelos et al., 2011b), leading to the hypothesis that the Lusitanian toadfish did not evolve seasonal changes in auditory function because the species is acoustically active throughout the year (Vasconcelos et al., 2011a).

The associations between E2 level and hearing metrics were primarily related to suprathreshold processing, rather than threshold shifts. The positive association between E2 level and threshold at

100–200 Hz could indicate that E2 adjusts frequency filtering properties of the auditory system to increase the critical ratio of the auditory filter around peak frequencies of male vocalizations (160–180 Hz). Indeed, E2 has been linked to temporal encoding ability in plainfin midshipman (Sisneros et al., 2004), and the effects of hormones and neuromodulators on auditory function have been found to exhibit stimulus specificity (e.g. Miranda and Wilczynski, 2009; Maruska and Tricas, 2011).

#### Auditory threshold

The lower auditory thresholds in females relative to males is consistent with female behavioral responsiveness in phonotaxis trials (Rollo et al., 2007) and supports the hypothesis that females rely on acoustic signals to locate nesting males. In particular, heightened auditory sensitivity at higher frequencies (above 300 Hz) could give females an advantage in detecting male vocalizations in shallow waters, where propagation of low frequency components is limited (Mann and Lobel, 1997; Bass and Clark, 2003). Round goby nests have been found as deep as 11 m (Wickett and Corkum, 1998) and as shallow as 1 m (Charlebois et al., 1997), and thus enhanced detection of higher frequencies could significantly increase detection distance. A similar scenario occurs in *Porichthys notatus*, where temporal encoding of the upper harmonics of male vocalizations is thought to facilitate female localizing of males calling in shallow water (Sisneros, 2009). However, unlike the relatively linearly shaped plainfin midshipman audiogram (Rohmann and Bass, 2011), the round goby audiogram peaks at 300 Hz and then declines as frequency increases, a pattern that has been observed in other fishes (e.g. *Coregonus nasus*, *Cottus ricei*) (Mann et al., 2007).

As males and females in the current study overlapped little in body size and length, it is possible that the observed sex differences in auditory function are due to non-sex-specific growth-related effects. Nonetheless, such a mechanism would still have sex-specific implications, as 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, body size is likely an important factor influencing male reproductive success, as large

Table 2. Summary of effects on suprathreshold AEP measures

Measure	Acoustic stimuli	No. fish tested	Sex	Receiver conditions		
				Male reproductive state	Female reproductive state	Female E2
Amplitude (p–p)	Pulse	N=14 NRF, 13 RF, 16 NRM, 11 RM	$t_{52}=0.43$ , $P=0.67$	$F_{1,22}=1.80$ , $P=0.19$	$t_{25}=1.61$ , $P=0.12$	+ $F_{1,12}=7.73$ , $P=0.017$ , $R^2=0.39$
	Pulse (r.m.s.)	N=13 NRF, 11RF, 15 NRM, 11RM	$t_{48}=0.15$ , $P=0.88$	$t_{24}=1.34$ , $P=0.20$	$t_{19,1}=0.57$ , $P=0.58^{\dagger}$	$F_{1,12}=3.96$ , $P=0.07$ , $R^2=0.25$
	100–200 Hz	N=12 NRF, 10 RF, 14 NRM, 11 RM	$t_{48}=1.08$ , $P=0.29$	n.s. omnibus* Mass: $F_{1,22}=2.83$ , $P=0.11$ TL: $F_{1,22}=2.76$ , $P=0.11$	n.s. omnibus $F_{1,20}=0.31$ , $P=0.58$	$F_{1,14}=4.49$ , $P=0.052$ , $R^2=0.49$
	300–600 Hz	N=12 NRF, 10 RF, 14 NRM, 11 RM	F>M $t_{48}=3.92$ , $P<0.001$	n.s. omnibus* Mass: $F_{1,22}=2.83$ , $P=0.11$ TL: $F_{1,22}=2.76$ , $P=0.11$	n.s. omnibus $F_{1,20}=0.31$ , $P=0.58$	$F_{1,14}=0.002$ , $P=0.96$
Latency	Pulse	N=14 NRF, 13 RF, 16 NRM, 11 RM	$t_{52}=1.32$ , $P=0.19$	$t_{25}=0.39$ , $P=0.70$	$t_{25}=0.64$ , $P=0.43$	+ $F_{1,12}=5.17$ , $P=0.042$ , $R^2=0.30$
	100–200 Hz	N=12 NRF, 13 RF, 14 NRM, 11 RM	n.s. omnibus $F_{1,45}=0.13$ , $P=0.72$	RM>NRM $t_{23}=2.77$ , $P=0.011$	n.s. omnibus $F_{1,23}=0.05$ , $P=0.82$	$F_{1,14}=0.80$ , $P=0.38$
	300–600 Hz	N=12 NRF, 13 RF, 14 NRM, 11 RM	n.s. omnibus $F_{1,45}=0.13$ , $P=0.72$	$t_{23}=0.92$ , $P=0.37$	n.s. omnibus $F_{1,23}=0.05$ , $P=0.82$	$F_{1,14}=0.65$ , $P=0.43$

\*ANCOVA including body mass or total length as a covariate;  $^{\dagger}$ unequal variance *t*-test. The + symbol indicates a positive relationship.



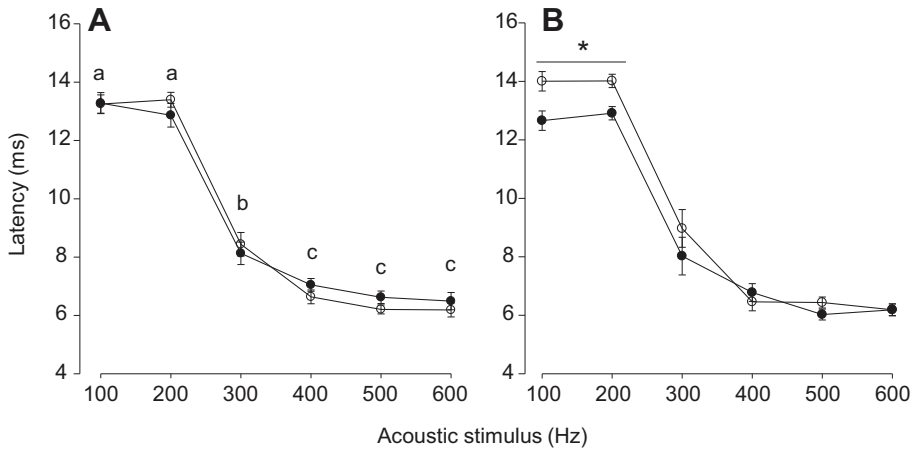


Fig. 6. (A) Latency of AEPs in response to tonal stimuli, grouped by sex (males, open circles; females, filled circles). (B) Male response latency grouped by reproductive condition (reproductive males, open circles; non-reproductive males, filled circles). Sex (A) and reproductive condition differences (B) are indicated by asterisks above frequency categories. 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 the effect of frequency on response latency. Frequencies possessing different letters are significantly different.

males are more likely to successfully occupy nests than are smaller males (Stammler and Corkum, 2005).

#### Latency

Latency decreased as frequency increased, which is a pattern that has been observed in other fish AEP studies (Kenyon et al., 1998; Ladich and Yan, 1998; Higgs et al., 2003). The neural regions responsible 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). Studies using both 'whole-field' AEPs and single unit recordings have found that delayed auditory latencies are associated with poor performance in other auditory measures, which were the basis of our initial predictions (Lucas et al., 2002; Goense and Feng, 2005; Maruska and Tricas, 2011). However, to understand the significance of these results, it is also crucial to evaluate latency concurrently with additional hearing measures. Indeed, the longest latencies in the current study occurred at frequencies of best auditory sensitivity (100–200 Hz), indicating that long latency may not necessarily indicate poor overall auditory function.

In the current study, E2 was positively associated with pulse latency, and reproductive males had longer latencies than non-reproductive males at 100–200 Hz. In human auditory brainstem response (ABR) studies, increased ABR latencies in females have been associated with high levels of estrogen in the ovulatory phase of the reproductive cycle, which is when females also exhibit the greatest capacity for auditory sensitivity and frequency discrimination (McFadden, 1998; Walpurger et al., 2004). A similar phenomenon may occur in female round goby, where a positive association between AEP latency and E2 indicates a link between AEP latency and complexity of auditory signal discrimination. The latency shift observed in reproductive males at 100–200 Hz was not associated with changes in other hearing parameters at these frequencies; thus, the functional significance of this difference will require further study to aid interpretation.

#### Amplitude

The higher amplitudes in females at high tone frequencies indicate that sex differences in auditory function are extended to suprathreshold processing rather than being present only as threshold differences. Amplitudes of AEP responses should covary with the number of neurons synchronously firing in response to the acoustic stimulus (Hall, 1992) and thus indicate the salience of the acoustic stimulus in the brain. Suprathreshold AEP amplitudes have been

linked to acoustic discrimination in songbirds (Lucas et al., 2002; Lucas et al., 2007), although precise measures of frequency selectivity, phase locking, and/or pitch tracking are required to definitively address these correlates (e.g. Henry and Lucas, 2010). The restriction of the difference to high frequencies further highlights the potential importance of processing high frequency auditory content for females, also supporting the hypothesis of changes in auditory function to facilitate nest localization in shallow waters. Additionally, the observed increase in pulse response amplitude in relation to female E2 level further highlights the importance of acoustic detection in females for reproductive purposes.

#### Hair cell density

Hair cell densities were higher in females than in males, indicating that differences in hair cell number could contribute to the observed sex differences in auditory function in round goby. In plainfin midshipman, increases in saccular hair cell density have been shown to correlate with seasonal changes in auditory sensitivity in females (Coffin et al., 2012), suggesting hair cell density can be a direct driver of sensitivity differences and represent a condition-independent adaptation in females for localizing breeding colonies. Additionally, Coffin and colleagues found a negative correlation between saccule hair cell density and fish size, with reproductive females tending to be slightly larger than non-reproductive females (despite reproductive females having lower auditory thresholds) (Coffin et al., 2012). In a

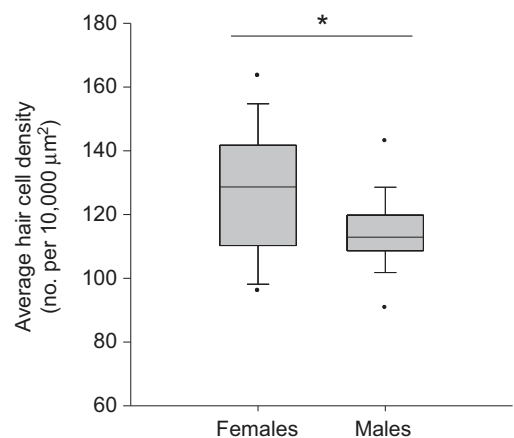


Fig. 7. Box plots indicating average saccule hair cell density in males and females. Box whiskers indicate the 10th and 90th percentiles. Dots indicate outliers.



separate study on the plainfin midshipman, Sisneros and Bass found that increasing body length was associated with decreases in threshold at the best frequency (Sisneros and Bass, 2005). Taken together, previous work in plainfin midshipman and our current findings indicate that although hair cell density may be related loosely to auditory function, additional factors besides hair cell density (e.g. otolith shape, kinocilium morphology, hair cell electric resonance) may also be important factors in auditory function.

The results from the current study contribute to our understanding of the link between hair cell number and auditory function in teleosts. Hair cell number increases with age in fishes (Corwin, 1983; Popper and Hoxter, 1984; Lombarte and Popper, 1994), but relatively few studies have directly related hair cell number to measures of auditory ability (but see Corwin, 1983; Higgs et al., 2002; Coffin et al., 2012). A lack of change in auditory thresholds across development has been observed in a number of studies (Higgs et al., 2002; Mann et al., 2009; Belanger et al., 2010) and this lack of change has been explained by hair cell addition matching the growth of the ear, resulting in a constant degree of hair cell stimulation (Rogers et al., 1988; Popper et al., 1988; Higgs et al., 2002; Higgs et al., 2003). However, this model assumes a relatively constant density of hair cells. It will be crucial for future studies to distinguish between total hair cell number and hair cell density.

### CONCLUSIONS

The sexually dimorphic and condition-dependent auditory function observed in the current study highlights the potential for sexual selection to act on sensory systems to facilitate reproductive behaviors. Additionally, the results indicate that the round goby is a useful model system for examining links between sensory function and reproductive behaviors. The reproductive state-dependent sensory flexibility recently observed in the pheromonal communication system of round goby (Belanger et al., 2004; Belanger et al., 2007) can now be extended to the acoustic modality. Olfactory and auditory function in the round goby clearly varies depending on multiple aspects of receiver condition, which can have profound implications for reproductive behaviors (Gammon et al., 2005; Rollo et al., 2007; Kasurak et al., 2012). More generally, several hypotheses generated from the current study may be used to better understand the reproductive ecologies and physiological mechanisms that give rise to reproductive state-dependent sensory flexibility and sex differences in sensory systems.

### LIST OF ABBREVIATIONS

AEP	auditory evoked potential
E2	17 $\beta$ -estradiol
GSI	gonadosomatic index
NRF	non-reproductive female
NRM	non-reproductive male
p-p	peak to peak amplitude
r.m.s.	root mean square amplitude
RF	reproductive female
RM	reproductive male
TL	total length

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