Long duration advertisement calls of nesting male plainfin midshipman fish are honest indicators of size and condition

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ABSTRACT

The plainfin midshipman fish (*Porichthys notatus*) has long served as a model organism for neuroethology research on acoustic communication and related social behaviors. Type I or "singing" males produce highly stereotyped, periodic advertisement calls that are the longest known uninterrupted vertebrate vocalizations, lasting up to two hours in duration. Despite the extensive literature on the acoustic behaviour of this species, it remains unclear whether reproductive males signal their quality via their highly energetic, multiharmonic advertisement calls. Here, we recorded the advertisement calls of 22 reproductive type I males at night in a controlled laboratory setting in which males were housed in artificial tanks maintained at a constant temperature (13.9 + 0.3°C). The duration of the advertisement calls from type I males was observed to increase from the first call of the night to the middle call after which call duration remained steady until the early morning hours and first light. A strong positive correlation was observed between loudness (SPL and maximum SPL) of the advertisement call and body size (mass and

standard length; $r_s > 0.8$). In addition, an asymptotic relationship was observed between the harmonic frequencies (F_0-F_{10}) of the advertisement calls and male body condition, with harmonic frequencies initially increasing with body condition but then plateauing at higher body condition. Taken together, our results suggest that type I male advertisement calls provide reliable honest information about male quality regarding size and body condition. Such condition dependent information of calling males could potentially be used by receptive females to help facilitate mate choice decisions.

INTRODUCTION

Animal signals provide information from one animal to another and often convey information that is the result of natural selection on the sender to help the receiver make a decision, which on average benefits both parties (Bradbury and Vehrencamp, 2011; Smith and Harper, 2003). The use of acoustic signals to convey social information is widely used by animals during reproductive and social behaviours. Acoustic signals can provide a variety of sender information to the receiver including species identification, sex, individual identity, sexual receptivity, motivation and condition-dependent information used in sexual selection such as age, body size and condition (Bradbury and Vehrencamp, 2011). In the context of reproductive behaviours, acoustic signals are often used as advertisement signals to attract mates (Amorim et al., 2015; Brillet and Paillette, 1991; Catchpole and Slater, 2003; Charlton et al., 2007; Gerhardt and Huber, 2002; Ryan, 1985a). Most commonly, advertisement signals are produced by males to attract receptive conspecific females for solicitation, courtship, mating, copulation/gamete release, and postmating announcements (Bradbury and Vehrencamp, 2011).

Acoustic advertisement calls can be physiologically expensive to produce. Increased calling activity has been known to decrease lipid content and growth rate in frogs (Given, 1988), while increasing oxygen consumption in frogs (Given, 1988) and crickets (Prestwich and Walker, 1981). The energy efficiency of call production can be extremely low, ranging from 0.05-3.4% in insects (Kavanagh 1987; Nally and Young, 1981), 0.05-1.2% in frogs (Ryan, 1985a), and 2% in birds (Brackenbury, 1980), making them good candidates as "honest signals" that can convey condition-dependent information about the sender. The calling effort (percentage of time spent calling relative to the total calling period) of acoustic advertisement calls is known to directly reflect a sender's energy expenditure in diverse taxa such as frogs (Bucher et al., 1982; Taigen and Wells, 1985), birds (Eberhardt, 1994), and insects (Nally and Young, 1981). Higher calling effort is associated with senders that are in better condition, or have greater energy reserves in several vertebrate clades such as fishes (Amorim et al., 2010; Pedroso et al., 2013), birds (Reid, 1987), and frogs (Ziegler et al., 2016). Calling effort can be enhanced by increasing either calling rate, call duration, or both. Other signal characteristics of advertisement calls that are condition dependent include the amplitude or loudness of the signal and its spectral properties such as the fundamental frequency and associated harmonics. Loudness of advertisement calls have been associated with body size in certain fishes (Amorim et al., 2013; Lindström and Lugli, 2000), birds

(Cardoso, 2010), and insects (Gray, 1997). In addition, larger individuals produce calls with lower fundamental frequencies and harmonics in fishes (Amorim et al., 2013; Myrberg Jr et al., 1993), frogs (Davies and Halliday, 1978; Robertson, 1986), birds (Mager et al., 2007; Marcolin et al.), and mammals (Reby and McComb, 2003; Vannoni and McElligott, 2008). In general, larger animals tend to possess larger sound producing organs that produce louder calls containing lower fundamental frequencies and harmonics.

Fish likely represent the largest taxon of sound producing vertebrates and the production of acoustic advertisement signals is an important component of mating behaviour in at least 800 species (Ladich, 2015; Radford et al., 2014). Teleost fishes have evolved a diversity of sound producing organs and can generate sound by plucking enhanced tendons (Kratochvil, 1978; Ladich, 2004), rubbing bony elements such as teeth or enhanced fin rays (Fine and Parmentier, 2015), stridulating the entire pectoral girdle or other hard skeleton parts (Bertucci et al., 2014; Colleye et al., 2012; Parmentier et al., 2007), or by vibrating the swim bladder with specialized sonic muscles (Bass and McKibben, 2003; Fine and Parmentier, 2015; Fine et al., 2001). One sound producing fish that become a good model organism for investigating the neural mechanisms of vocal production and auditory reception shared by all vertebrates is the plainfin midshipman, *Porichthys notatus*, which is a vocal marine teleost that uses social acoustic signals for communication during reproductive and social behaviours (Bass and McKibben, 2003; Bass et al., 1999; Fay and Simmons, 1999; Forlano et al., 2015). Midshipman are nocturnally active and produce a relatively simple repertoire of social acoustic signals that include "grunts", "growls" and "hums" by using sonic muscles to vibrate their swim bladders (Bass et al., 1999; Sisneros, 2009). All three adult midshipman sexual phenotypes (females and males: types I and II) are capable of producing short-duration, broadband agonistic grunts, but only type I nesting males can produce trains of grunts, long duration agonistic growls, and the multi-harmonic advertisement calls or "hums" (Brantley and Bass, 1994; McKibben and Bass, 1998; Sisneros, 2009).The advertisement calls of midshipman are unique among vertebrates in that they are continuous, highly stereotyped and long in duration, ranging from 1 minute to a couple of hours (Bass et al., 1999). Females rely on their auditory sense to detect and locate "singing" males that produce the multi-harmonic advertisement calls during the breeding season. Reproductive females become adaptively tuned to detect the dominant harmonic components of male advertisement calls due to seasonal increases in estrogen levels prior to the breeding season (Sisneros and Bass, 2003; Sisneros et al., 2004a; Sisneros et al., 2004b). This seasonal enhancement in auditory sensitivity to the higher dominant harmonics of the advertisement call may be important for conspecific detection and localization in shallow water acoustic environments and for the perception of auditory information used in mate choice decisions. Despite this seasonal shift in female auditory tuning to become better suited to detect male vocalizations, it remains unknown whether the male advertisement call contains condition dependent information about the sender that could potentially be used in decisions of mate choice.

The primary goal of this study was to characterize the bioacoustics of male advertisement calls and determine if there are any relationships between spectro-temporal features in the advertisement calls and condition dependent morphometrics of calling type I males. We hypothesize that the multiharmonic advertisement calls of type I males are honest indicators of morphometric parameters which reflect male quality, such as size and body condition. Based on previous bioacoustics studies, we predict a positive correlation between calling effort and body condition (Pedroso et al., 2013). We also expect a negative correlation between harmonic frequencies and body size, and a positive relation between loudness and body size (Amorim et al., 2013). Additionally, we investigate if there are temporal rhythms/ patterns in male calling behaviour. We interpret our findings as they relate to the possible use of condition dependent information in midshipman acoustic communication during social and reproductive behaviours.

MATERIALS AND METHODS

Animals

Type I male plainfin midshipman were captured during periods of low tide when male nests were exposed at Seal Rock, Brinnon, WA, U.S.A in the late spring-summer breeding seasons (April-July) of 2019 and 2020. Animals were transported to the University of Washington and housed in artificial saltwater tanks maintained at a salinity of 26-28 parts per thousand (ppt) and a controlled temperature of $13.9 + 0.3°C$ SD using aquarium chillers. The fish were acclimatized to 14:10 hours of light:dark cycle by turning the lights off at 20:00 h and turning them on at 06:00 h, approximating the ambient day-night cycle during the nesting period in the wild. Males were fed small pieces of thawed frozen shrimp 2 times per week.

Experimental setup and acoustic recordings

Two artificial nest chambers were constructed by placing terracotta pot plant saucers (diameter = 25 cm, height= 3.5 cm) over two bricks ($5.7 \times 5.7 \times 22$ cm^3), in two separate 50-gallon tanks ($121 \times 32 \times 43$ cm^3). A brick was placed on top of the pot plant saucer to prevent it rattling when the male vocalized (Fig. 1). The nest chambers were filled with sand to entice the males to excavate and build their own nests. Three to five type I males were added to each tank with the nest chambers. We waited 2-3 days for the nest to be occupied. If no male occupied the nest during these days, either more males were added into the tank or the males residing in the tank were replaced with new individuals. We observed that a strong indicator of the propensity to produce advertisement calls during a given night was the tendency of the resident nesting male to float up against the pot plant saucer (ceiling of nest chamber), presumably due to the increase in swim bladder volume. As soon as this floating behaviour was observed, non-nesting males were removed from the tank to ensure that any recorded call was generated by the nesting male. Floating behaviour generally continued even after a night of calling. An HTI-99-HF hydrophone

(Sensitivity: -204 dB re: 1V/ µPa; Frequency response: 2 Hz - 125 kHz) (High Tech Inc., Long Beach, MS) was positioned above the center of the nest (Fig. 1). The hydrophone was connected to a recorder (Zoom H2 digital recorder, Zoom, Hauppauge, NY). Calls were recorded overnight onto an SD card at a sampling frequency of 44.1 kHz and a bit depth of 16. Calls were recorded from a total of 22 type I males, 8 males recorded during the summer of 2019, and 14 males recorded during the summer of 2020. The hydrophone was positioned a fixed distance of 1 cm above the center of the artificial nest in the recordings conducted in 2020 ($N = 14$), whereas in 2019 the hydrophone was positioned at a variable distance of ~1-10 cm above the nest ($N = 8$) (Fig. 1a). Additionally in 2020, the hydrophone-recorders were calibrated using a pistonphone (Type 42AC, G.R.A.S. Sound & Vibration, Holte, Denmark). Background noise in the tanks was primarily due to an aquarium chiller, which turned on and off periodically, and a submersible water pump that stayed on throughout the course of the recording period. Background noise level was 122 dB 1µPa RMS with the chiller on and 113 dB re 1µPa RMS with the chiller off.

Morphometric parameters

After a single night of calling, males were sacrificed in a saltwater bath containing excess 10% benzocaine. In 2019 ($N = 8$ males), swim bladders were extracted from calling type I males for an alternate set of experiments. Body mass measured to the nearest 0.1 g did not include the mass of the swim bladder (but did include the sonic muscles). Body mass without the swim bladder is highly correlated to total body mass ($R^2 \approx 1$; data from 2020, $N = 14$ males; Fig. S1). To maintain consistency between the two summers, all subsequent references to body mass excludes the mass of the swim bladder. We measured standard length to the nearest 0.1 cm. The sonic muscles were carefully removed from the attached swim bladder using scissors and forceps and their mass was measured. In 2020 ($N = 14$ males), we also measured the mass of the swim bladder from calling type I males. The swim bladder comprised 1.91 + 0.3% SD of the total body mass ($N = 14$; data from 2020). Mass of the gonads were measured and used to calculate the gonadosomatic index (GSI), the ratio of gonad mass to somatic mass expressed as a percentage computed by the formula 100*[Mass of gonads/(Body mass-Mass of gonads)].

Body condition was estimated using two measures, the residuals of the regression between the common logarithm (log) of body mass (in g) vs log of standard length (in cm) (COND), and Fulton's condition factor (K) which was computed using the formula 100*(Body mass/Standard length^3). Both COND and K are indirect measures of energy reserves in fish (Chellappa et al., 1995; Sutton et al., 2000) and have been used to assess body condition in the plainfin midshipman (Bose et al., 2018; Sisneros et al., 2009) and related toadfish (Amorim et al., 2010; Amorim et al., 2016). In 2020 ($N = 14$ males), we measured the mass of the liver and used it to compute the hepatosomatic index, the ratio of liver mass to

somatic mass expressed as a percentage computed by the formula 100*[Mass of liver/(Body mass-Mass of gonads)]. The hepatosomatic index also reflects energy reserves in some fish species (Chellappa et al., 1995).

Spectro-temporal features of the advertisement call

Type I males produce long-duration, multi-harmonic advertisement calls that are highly periodic with a fundamental frequency of around 80-100 Hz and contain harmonics up to 1000 Hz (Fig. 2). For every advertisement call, the duration was measured using Raven pro v1.5 (Center for Conservation Bioacoustics, Cornell lab of Ornithology, Ithaca, NY). Type I males produced advertisement calls almost exclusively during the night phase of the photoperiod between 8 pm and 6 am, when the lights were turned off. Calling rate was defined as the number of calls per hour. For each male, calling rate was computed as the ratio of the total number of calls produced overnight to the number of hours present in the dark photoperiod (10 hours). Calling effort for each individual (percentage of time spent calling) was computed by calculating the ratio of the total time spent calling (in hours) to the total number of hours present in the dark photoperiod (10 hours) and multiplying the ratio by 100.

Each advertisement call contained an initial transient phase at the beginning of the call that was marked by a sharp rise and fall in amplitude. After the initial transient phase of the call, the amplitude becomes relatively stable for the remainder of the advertisement call (Fig. 3). The transient phase of the call was the segment that contained the largest amplitude of the signal. Recordings from calling type I males in 2020 (N =14) were calibrated using a pistonphone. For every call recorded in 2020 we selected ~1s duration call segments of the transient phase where the amplitude was greatest using Raven pro v1.5 (Fig. 3). The root mean square (RMS) amplitude of this transient phase segment was computed to estimate the maximum amplitude produced during the call. The RMS amplitude of the remaining stable portion of the advertisement call was also computed. Sound pressure level (SPL) in dB was computed from the rms pressure amplitude using the following equation,

$$\text{SPL (dB)} = 20* \ \log_{10}\left(\frac{p}{p_{ref}}\right) \tag{1}$$

Where p= RMS pressure amplitude, and p_{ref}= 1 µPa. Sound pressure level of both the maximum amplitude of the call during the transient phase (henceforth called maximum SPL) and the stable portion (henceforth referred to as just SPL) of the advertisement call was computed for each call produced by the calling type I males. SPL and maximum SPL reflect the loudness of the advertisement call.

We extracted 1-minute audio clips from the stable portion of the advertisement signal for each call. These 1-minute segments were used in the analysis of the following features: the first eleven harmonics (F_0-F_{10}), harmonic decay rate and aggregate entropy. Harmonics, ranging from the fundamental frequency (F_0) to the eleventh harmonic (F_{10}), were estimated by manually marking the peaks in the power spectrum, which was generated using custom code in MATLAB (Mathworks, Natick, MA, USA). The amplitude of each harmonic tends to decrease exponentially with harmonic number (Fig. S2). Exponential functions of the form $y= a*e^{-bx}$ were fit to a plot of amplitude versus harmonic number (0-10) in MATLAB, where a and b are positive constants (Fig. S2). The parameter b represents the rate of decline in harmonic amplitude with increase in harmonic number, with larger values representing a steeper decline in the amplitude of higher harmonics relative to the fundamental frequency. We term the parameter b as "harmonic decay rate". An alternative way to measure how evenly acoustic energy is distributed among harmonic frequencies is through aggregate entropy (H) (Charif et al., 2010). H was computed using the following equation:

$$H= \sum_{f=f1}^{f2} \frac{E_{bin}}{E} * \log_2 \left(\frac{E_{bin}}{E} \right)$$

(2)

Where, f1 and f2 are lower and upper frequency bounds on the spectrogram, E_{bin} is the energy in each bin, and E is the total energy summed over each bin. Aggregate entropy was computed for each advertisement call from spectrograms using Raven pro v1.5. The following settings were used to generate the spectrogram of the call: a Hann window with 65525 samples and a 3 dB filter bandwidth of 0.968 Hz, a hop size of 32763 samples with 50% overlap, frequency grid with grid spacing 0.673 Hz and DFT size 65536. A signal dominated by the fundamental frequency will have low aggregate entropy whereas a signal with more even energy distribution among all the harmonic frequencies will have higher aggregate entropy.

To determine if call duration changes during the night, we used a Friedman test to compare the durations of the first, middle, and final calls produced during the night. Conover post-hoc tests with Bonferroni corrections were used to test for pairwise differences. The criterion for significance α was set to 0.05. If the total number of calls produced by an individual (n) was an odd number, then the $[(n+1)/2]^{th}$ call was considered as the middle call and its duration was noted. If the individual produced an even number of calls, the middle calls comprised both the $(n/2)^{th}$ call and the $[(n/2)+1]^{th}$ calls. The duration of the "middle" call was then computed as the average of the durations of the $(n/2)^{th}$ and the $[(n/2)+1]^{th}$ calls. Mean call duration was highly variable across animals. To facilitate comparison of temporal patterns in duration across animals, call duration was normalized for each animal by dividing the duration of each call by the length of the longest call produced by that animal. A non-parametric test was used for pairwise

comparisons because the assumption of normality was violated for the distribution of duration of the first call (Shapiro-Wilk test; $W = 0.84$; $P < 0.01$). One individual was excluded from the analysis comparing the durations of the first, middle, and final calls as it produced two calls during the recording period.

Correlating morphometrics with spectro-temporal features of the advertisement calls

We measured pairwise Spearman's rank correlation coefficients (r_s) between morphometric parameters and spectro-temporal call features. A t-test was used to determine if r_s was significantly different from zero ($\alpha = 0.05$) (Zar, 1972). The morphometric parameters are mass, standard length, body condition measures (COND and K), sonic muscle mass, swim bladder mass, liver mass, and hepatosomatic index. Call features computed are calling rate, calling effort, and the means of duration, sound pressure level, maximum sound pressure level, fundamental frequency (F_0), harmonic decay rate (b) and aggregate entropy (H).

RESULTS

Advertisement calling activity and temporal patterns of call duration

The calling type I males had a size range of 18.1 cm to 27.2 cm standard length (SL) with a mean SL of 23.1 + 3.4 cm SD, mean body mass (BM) of 173.5 + 74.5 g SD, mean COND of 0.00 + 0.05, mean Fulton's condition factor (K) of 1.32 + 0.15 g/cm^3 SD, mean gonadosomatic index of 2.15 + 0.76 SD, mean swim bladder mass of 3.60 + 1.41 g SD, mean sonic muscle mass of 2.53 + 0.93 g SD, mean liver mass of 3.29 + 1.25 g SD, and mean hepatosomatic index of 1.85 + 0.40 g SD. A total of 408 calls were recorded from 22 type I males. The time to start calling after the onset of the dark photoperiod was variable, ranging from around 15 minutes to almost 8 h (mean=1h:46 min; SD=1h:59 min). One male began calling approximately half an hour before the onset of the dark photoperiod. The number of calls produced overnight ranged from 2 calls to 80 calls (mean number of calls per night = 19 + 18). The calling rate (calls per hour) varied among the type I males that ranged from 0.2 calls per hour to 8 calls per hour (mean call rate = 1.9 + 1.8 SD calls/h). Call duration ranged from 6.5 s to 1 h 34 min (Mean call duration= 9 min:46 s; SD= 10 min:40 s). Calling effort ranged from ~2% of the dark photoperiod (20 min) to ~65% (6 h 30 min) with mean calling effort being 31 + 20% SD which equates to 3 h 6 min + 2 h SD of time spent calling each night on average. The duration of the transient phase ranged from 1.0 s to 109.5 s, with mean duration being 28.1 + 17.4 s SD. For the calls recorded from males in 2020 ($N = 14$), the sound pressure level (SPL) of the transient phase exceeded the SPL of the steady phase by an average of 1.9 + 1.7 dB SD.

The duration of the calls produced by vocally active type I males was observed to increase over the course of the night and then remain roughly consistent until the early morning hours (Fig. 4a-d, also see fig. S3). There was a significant main effect of call position (first, middle, or final) on call duration

(Friedman test: $\chi^2(2) = 26.95$, Kendall's $W = 0.58$, $N = 21$, $P < 0.001$). Duration of the middle call and final calls were significantly greater than the duration of the first call (Conover post hoc test: $N = 21$, $P < 0.001$ for both comparisons; Fig. 4e). Thus, our data shows that type I males, on average, initially produce calls that are shorter in duration at the beginning of the night and as the night progressed call duration increased and remained steady until the males stopped calling in the early hours of the morning.

Spectro-temporal characteristics of advertisement calls predict male morphometrics

We observed strong positive correlations between body size measures and loudness of the advertisement calls. Sound pressure level (SPL) was positively correlated with body mass (BM) ($r_s = 0.81$, $N = 14$, $P < 0.001$; Fig. 5a), and standard length (SL) ($r_s = 0.83$, $N = 14$, $P < 0.001$; Fig. 5b) (Table 1). Maximum SPL was also positively correlated with BM ($r_s = 0.81$, $N = 14$, $P < 0.001$; Fig. 5c), and SL ($r_s = 0.84$, $N = 14$, $P < 0.001$; Fig. 5d) (Table 1). Thus, advertisement call loudness was a strong predictor of body size for the calling type I males. The measures of advertisement call loudness (SPL and maximum SPL) were also positively correlated with swim bladder mass, sonic muscle mass, and liver mass ($r_s = 0.56$-0.85, $N = 14$, $P < 0.05$) (Table 1).

The fundamental frequency and higher harmonics (F_{0-10}) of the advertisement call increased initially with increasing body condition but then plateaued at higher body condition of calling type I males (Fig. 6). Approximately 50% of the variation in the plots of call harmonics vs body condition can be explained by fitting an asymptotic regression model of the form $y = F_{AS} - (F_{AS} - I)*e^{-kx}$ where y= Harmonic frequency (F_{0-10}), x= Body condition (COND or K), F_{AS} is the asymptotic frequency at higher body condition, I is the intercept, and k represents the rate of exponential decay (Fig. 6, Table 2). An asymptote in the relationship between the harmonic frequencies with body condition occurred at a threshold body condition of approximately 0 (COND) and 1.3 g/cm^3 (K) (Fig. 6). F_0 was also significantly positively correlated with K ($r_s = 0.48$, $P < 0.05$) but not COND (Table 1). Thus, the harmonic frequencies of the advertisement call serve as a predictor of body condition, with males above a threshold body condition being able to produce calls with higher harmonic frequencies.

There was no correlation between measures of calling activity such as calling rate, call duration, and calling effort and the morphometric parameters examined. Since we demonstrated temporal patterns in call duration, only calls produced after the middle call were used to compute mean call duration to avoid the potential confounding effects of the temporal patterns in call durations as duration increased from initial to middle calls. Therefore, n/2 or (n+1)/2 calls were included in the computation of mean call duration if the total number of calls produced by the type I male (n) was even or odd respectively. Features measuring the energy distribution among harmonic frequencies, aggregate entropy (H) and harmonic decay rate (b) also did not show correlation with any of the morphometric parameters examined (Table 3).

DISCUSSION

The primary goal of this study was to characterize the advertisement calls of reproductive type I male midshipman and determine whether these calls contain condition dependent information about the sender that could potentially be used in mate choice decisions. In this discussion, we consider how the vocal activity patterns of type I males, the observed correlation between loudness of the advertisement calls and body size, and the asymptotic relation between harmonic frequencies and body condition may influence mate choice decisions in female plainfin midshipman.

Advertisement calling activity and temporal patterns of advertisement call duration

Despite occupying artificial nests in a laboratory setting, type I males called for an average period of 3 hours during the dark photoperiod. Mean call duration was approximately 10 minutes. The average calling period and call duration is consistent with previous studies on captive type I male midshipman (Brantley and Bass, 1994; Feng and Bass, 2016; Ibara et al., 1983). Measures of vocal activity such as call duration, rate, and effort did not correlate with the morphometrics of mass, standard length, or body condition. This is in contrast to other studied fishes such as the painted goby where calling rate reflects body condition (amount of energy reserves) (Amorim et al., 2013) and the Lusitanian toadfish, where both calling rate and calling effort positively correlate with body condition (amount of energy reserves) (Amorim et al., 2010). In these species, the advertisement calls are quite short in duration being ~0.05 s in the painted goby and ~0.6 s in the Lusitanian toadfish and therefore it is likely that females can directly assess these quantities. However, calling rate and effort cannot be directly assessed by female midshipman as call duration is of the order of minutes or hours and therefore these features may play a limited role in female midshipman mate choice decisions. An increase in calling activity over the course of the night could confer fitness benefits to the type I male by increasing the probability of detection by females. However, we did not observe any correlation between morphometric indicators of reproductive potential such as body condition and size, and any measures of vocal activity. It is possible that the artificial captive conditions in the laboratory may have potentially affected the natural calling activity of nesting type I males. It should also be noted calling activity was recorded over the course of a single night. Monitoring calling activity over a longer duration such as weeks or even the course of the entire breeding season could still reveal a correlation between measures of calling activity and morphometric parameters. However, calling activity appears to be inconsistent across nights, at least in captive conditions (Fig 3, Feng and Bass, 2016), strengthening the argument that there is no correlation between measures of calling activity such as call duration and effort and morphometric parameters predicting fitness or reproductive potential. Future studies that characterize the vocal activity of type I males in the natural nesting environment may provide helpful insight in determining whether the calling activity observed in the laboratory reflects the natural calling activity of nesting males in the wild.

We observed that type I males almost exclusively called during the dark photoperiod. In a previous study, Feng and Bass (2016) demonstrated that type I males primarily produced advertisement calls during the dark photoperiod and that they maintain a 24-hour circadian rhythm in calling activity, regulated by the hormone melatonin. Our study is consistent with their results, but we also show that there are temporal trends in calling activity during the night with call duration initially increasing and then remaining stable after the middle call of the night into the early morning hours. We should also note that other acoustic features (SPL, maximum SPL, F_0, H and b) of the initial calls did not differ from that of the middle or final calls (Table 3), indicating a lack of temporal patterns for these features.

Spectro-temporal characteristics of advertisement calls predict male morphometrics

We observed that larger males produced advertisement calls with greater amplitude. This observed increase in loudness with size is likely due, in part, to larger males having larger sonic muscles and larger swim bladders as shown here in the current study. Larger sonic muscles are capable of producing greater contraction forces to produce louder vocalizations, while larger swim bladders are better for transferring acoustic energy to the surrounding tissues and water. Thus, loudness of the advertisement call provides condition dependent information about the size of the caller. Previous work by McKibben and Bass (1998) revealed that female midshipman preferred louder calls when presented with two calls containing the same fundamental frequency. Females also preferred to spawn in the nests of the larger males, when allowed to choose between two males differing in body size (Bose et al., 2018). Furthermore, type I males with greater body size are known to father more offspring in the wild (Bose et al., 2018; Brown et al., 2021; DeMartini, 1988; Sisneros et al., 2009). Our study provides a positive link between body size and loudness, indicating that female preference for louder calls may also lead them into the nests of larger males. Nest takeovers are a common occurrence in the wild, with the winner likely feeding on the offspring of the displaced male (Cogliati et al., 2013). Therefore, choosing a large male for mating would likely improve female fitness, as larger males would be more likely to fend off smaller competitors and retain nests throughout the course of the breeding season.

The results from our study demonstrate that advertisement call loudness is related to the size of calling males, but call loudness decreases as a function of distance from the calling male. How might females use call loudness to access potential mates? One possibility would be to assess loudness at or in the nest, when females are very close or in physical contact with type I males. In a previous study, Brantley and Bass (1994) showed approximately 47% of the females that entered a type I male's nest left without spawning. One hypothesis for these results is that the loudness of the advertisement call produced by these type I males did not meet a loudness threshold required by females for mate selection and spawning. Further studies are required to test if the advertisement calls of type I males need to cross a loudness threshold in order to be selected by females for mating. The perception of advertisement call loudness by females in the natural environment may also be influenced by environmental factors such as

ambient sound levels and the distribution and attenuation of male advertisement calls in the rocky substrate environment where type I males "sing" and nest. Future studies that examine vocal signal propagation and loudness in the natural environment will be useful in determining the role of call loudness in mate selection and mate choice decisions by female midshipman.

Loudness or amplitude of the advertisement call maybe an important call attribute that females use in mate choice decisions to access large males as suitable fathers because type I males are the only providers of parental care after spawning. Sisneros et al. (2009) showed that larger nesting type I males (both greater in size and body mass) had a greater number of offspring in their nest at end of the nesting cycle, which suggests that larger males have greater spawning success, a greater capacity to care for more offspring, and the potential of having greater fitness. Similarly, Bose et al. (2018) showed in the field that male size and nest size were important correlates of reproductive success, but nest size was found to impose a limit on reproductive success regardless of male nest owner quality. Moreover, Bose et al. (2018) also showed in laboratory mate-choice experiments that females prefer larger males when nest size was held constant, while females showed no preference for larger nests when male size was held constant. Future studies that examine whether female preference for louder advertisement calls and larger males results in greater spawning success and fitness of calling type I males will be needed to determine if call loudness is important in female mate choice decisions.

Although the harmonic frequencies of the type I male advertisement calls were not found to be related to body size, we did find that the harmonics of the advertisement call were related to the body condition of the calling males. This relationship was best explained by an asymptotic regression model that showed that the harmonic frequencies of the advertisement call increased with body condition values up to a threshold of approximately 0 (COND) and 1.3 g/cm^3 (K) but not at higher body condition values where the harmonic frequencies plateaued (see Fig. 6). This inflection point (at ~ COND=0 and K=1.3 g/cm^3) in the relationship between the call's harmonic frequencies and body condition may represent a threshold for body condition which if crossed are indicative of high body condition or high quality of nesting type I males. Alternatively, this relationship may represent a threshold for body condition used by females to evaluate and avoid selecting mates that are in relatively poor body condition. Not surprisingly, Sisneros et al. (2009) reported that type I males with nests containing only fresh eggs from recent spawning (likely within 24 hours) had a minimum K value of 1.3 g/cm^3 and maximum K value of 1.9 g/cm^3 (mean K = 1.5 + 0.2 g/cm^3 SD; see Table I in Sisneros et al. (2009)). This body condition threshold or minimum K value of 1.3 g/cm^3 observed in successfully mated males may correlate with the production of an upper harmonic frequency limit of the male advertisement call at a given temperature and function to identify type I males that are in relatively high body condition. Body condition is an important morphometric and indicator of energy reserves for nesting type I males during breeding season when males are actively calling to attract mates but are slowly starving due to lack of food while in the nest. The data from our current study support the hypothesis that body condition may be an important criterion used

by females and can be obtained from the information in the harmonics of the male advertisement call. We should also note that body condition is also likely not the only criterion used by females for mate choice decisions. Brantley and Bass (1994) noted that type I males often stop calling shortly after females enter a male's nest during courtship. This suggests that other non-auditory sensory cues could be used by females to evaluate males such as the "fin quivers" or hydrodynamic fin movements produce by courting males that can be detected by the lateral line, or the olfactory signals produced by nesting males, and even perhaps the odors of eggs in the nest, which could potentially provide cues of mating success. Future studies that examine the attractiveness of different advertisement calls based on the harmonic frequency content related to body condition in behavioral two-choice playback experiments and whether other sensory cues are used during mating will be extremely useful in determining what information females use in mate choice decisions.

One critical factor that is important in determining the relationship between advertisement call harmonic frequencies and body condition is temperature. Previously, it has been demonstrated that the fundamental frequency and corresponding harmonics of the advertisement call vary linearly with temperature (Brantley and Bass, 1994; Halliday et al., 2018; McIver et al., 2014) and this is likely due to a temperature coupling mechanism of sender and receiver in the vocal communication system of the plainfin midshipman. Such temperature coupling mechanisms are known to occur in the vocal-acoustic systems of other vertebrates and in invertebrates (Brenowitz et al., 1985; Ladich, 2018; Pires and Hoy, 1992). McIver et al. (2014) showed that the fundamental frequency of the advertisement call varies linearly with temperature such that an increase in 1 C corresponds to a 5 Hz increase in fundamental frequency. Because the fundamental frequency and advertisement call harmonics vary with temperature, it was important to control and maintain a constant temperature in this study to reduce frequency variation in call's harmonics due to temperature. Midshipman research labs (including the Sisneros lab) have previously examined the potential relationship of the advertisement call harmonics with type I male morphometrics (e.g., size, body condition, etc.) but failed to find any significant relationships when not controlling for temperature. However, the current study is the first to control for temperature while examining such relationships. Across a temperature range of 12-18°C, females demonstrate in two choice experiments a strong preference for pure tones that match the fundamental frequency of average type I males calling at that temperature (McKibben and Bass, 1998). This fundamental frequency preference by females evokes strong phonotaxis to the simulated playback of a calling male and suggests at a given temperature there is optimum fundamental frequency and associated harmonics that influence mate choice decisions. When we maintained temperature constant at ~14°C in our recording tanks, we observed that males in relatively poorer body condition produced advertisement calls with lower fundamental frequencies and harmonics, which supports the hypothesis, at least at this temperature, that type I males in poorer body condition are unable to produce advertisement call frequencies that are preferred by females. Night temperatures recorded from the nesting grounds of plainfin midshipman at Seal Rock in Brinnon, WA, USA can range from 12-21°C during the breeding season (SB, unpublished

data). It remains to be seen whether the advertisement call frequencies of type I males are influenced by body condition at temperatures other than 14˚C in the wild. Future studies that examine the correlation of advertisement call frequencies with body condition at other temperatures would prove useful in determining whether body condition constrains the call's harmonic frequencies across the temperature range experienced by plainfin midshipman in the natural environment.

In addition to using their advertisement calls to attract mates, type I males may also employ nesting and chorusing strategies to enhance their probability of mating success. Based on the authors' personal field observations, nesting type I males do not appear to establish their nests randomly in the intertidal breeding zone. Instead, nests have commonly been observed to be clustered in groups, but it remains unclear what are the underlying factors responsible for the spatial distribution of the nesting clusters (e.g., the factors that may determine nest suitability such as the size, density, and sound propagating properties of the nests and how they are distributed in the intertidal zone). However, one potential explanation for the observed clustering of male nests in the intertidal nesting zone may be related to the "hotshot" hypothesis, which was originally proposed by Bradbury & Gibson (1983) to explain why males congregate at leks. According to the hotshot hypothesis, subordinate males cluster around highly attractive males to enhance their chances of interacting and potentially mating with females that are drawn to the "hotshots". Likewise, type I males that are smaller and/or in poorer body condition may establish their nest sites near larger and better conditioned type I males (i.e., "hotshots") during the breeding season as a strategy to enhance mating success. "Hot shot" nesting type I males may only be able to spawn with a limited number of females at a time, which may allow for nearby nesting males to court and spawn with other females when multiple females are attracted to the dominant or "hotshot" nesting male. An alternative, but not mutually exclusive, hypothesis for the clustering of nesting males in the intertidal environment maybe a hypothesis similar to the "female preference" hypothesis proposed by Bradbury (1981) that males cluster together because females prefer sites with large groups of males, where they can more quickly and/or more safely, compare the quality of many potential mates. In terms of plainfin midshipman, type I males may cluster in order to "sing" or produce their advertisement calls together, which can be commonly heard in midshipman field recordings that contain overlapping "hums" that interfere acoustically to produce acoustic beats (McIver et al., 2014). The chorus of "hums" produced by nesting males may function to attract females to a cluster of males and allow them to access a large group of males relatively quickly. If the attractiveness of chorus calls is greater than individual calls such as in the Eastern Gray Treefrog (Stratman et al., 2021), then such evidence would help support a female preference hypothesis for the clustering of nesting males. Future studies that examine the spatial distribution of the nests and the calling strategies of male midshipman in the natural environment would be informative in determining the adaptive strategies that type I males may use to potential enhance their reproductive success.

CONCLUSIONS

We show that nesting type I males primarily produce their multiharmonic advertisement calls during the dark photoperiod. Call duration steadily increased after the first call, rose to a peak value and then remained consistent until the early morning before light. Advertisement call loudness was strongly correlated with body size while the harmonics of the advertisement call initially increased with body condition up to a certain threshold body condition where it remained relatively constant and did not increase at higher body condition. Taken together our results suggest that the advertisement calls of type I male plainfin midshipman can potentially provide prospective mates with valuable condition dependent or "honest" information about both the size and body condition of the advertising male.

Competing interests

The authors declare no competing interests.

Author Contributions

Conceptualization: SB, JAS; Data curation: SB; Formal analysis: SB; Funding acquisition: JAS; Investigation: SB; Methodology: SB; Project administration: JAS; Resources: JAS; Software: SB; Supervision: JAS; Validation: SB, JAS; Visualization: SB, JAS; Roles/Writing - original draft: SB, JAS; Writing - review & editing: SB, JAS.

Data availability

Overnight acoustic recordings of the advertisement calls of type I males, and one minute call segments used for computing harmonic frequencies, aggregate entropy, and harmonic decay rate is available from the Dryad digital repository: https://doi.org/10.5061/dryad.pk0p2ngqf. Custom MATLAB codes used calibrate the sound files to compute SPL (in dB re: 1µPa), plot frequency spectrums and power spectral densities, and fit asymptotic regression models to plots of harmonic frequencies vs body condition are also available in same data repository.

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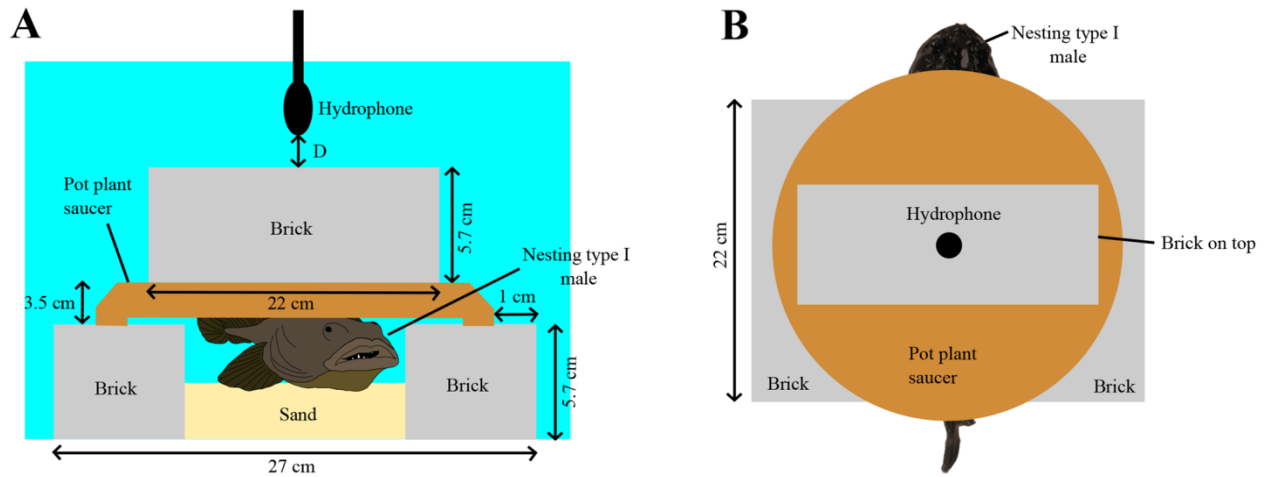
Figures

Figure 1. Illustrations depicting (A) frontal view and (B) top view of the artificial nest used to record the advertisement calls of type I males. During the acoustic recordings conducted in 2020 ($N = 14$), the hydrophone was placed at a fixed distance (D) = 1 cm on top of the artificial nest, approximately at the center. In 2019 ($N = 8$), the hydrophone was placed above the nest, but its position was more variable (~1-10 cm above the nest).
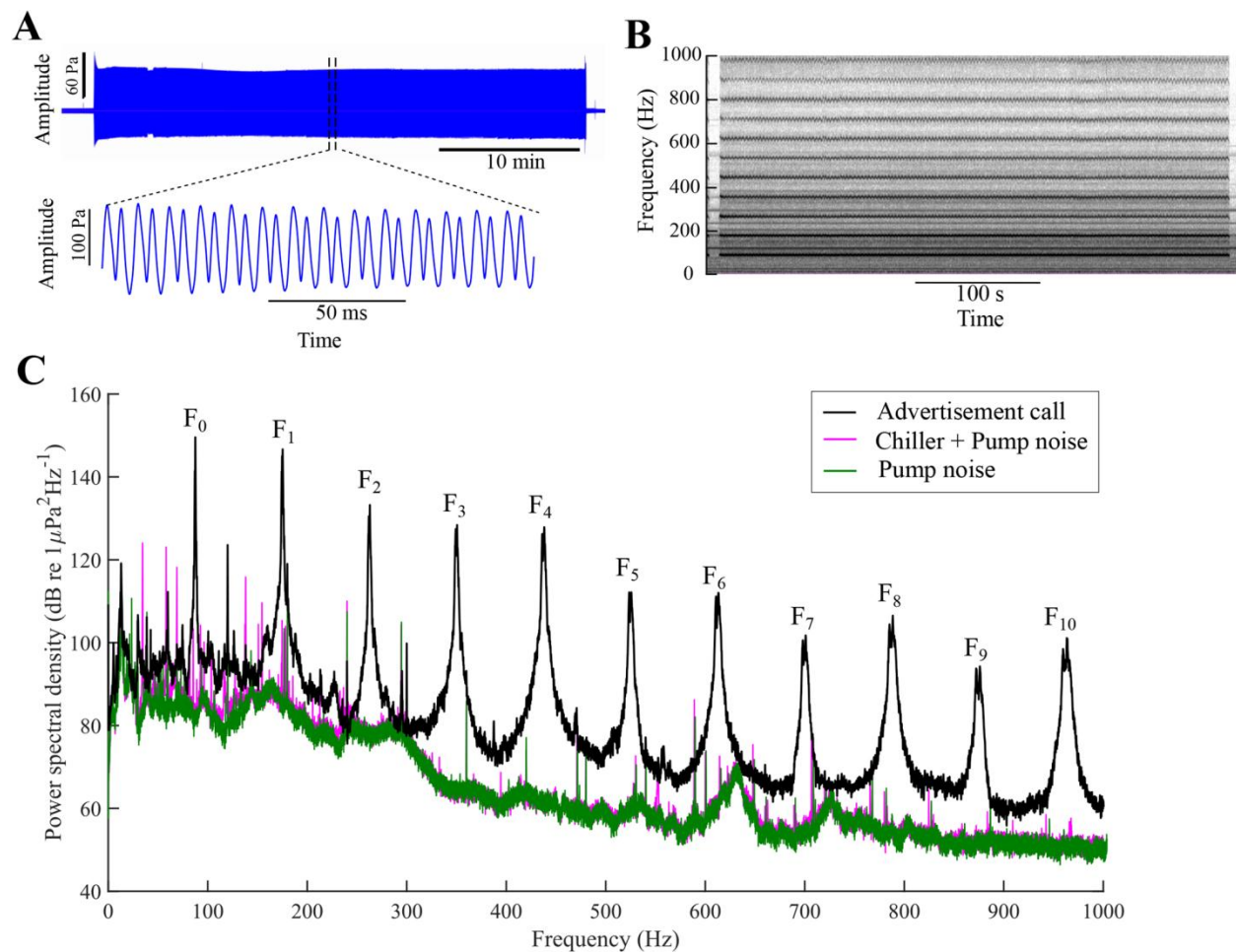
Figure 2. Advertisement calls of type I male plainfin midshipman. (A) Top- Oscillogram of a representative advertisement call which is ~35 minutes in duration; bottom- zooming into a small segment of the call demonstrates that the waveform is highly periodic in nature. (B) Spectrogram and (C) Power spectral density plot (black) of a representative advertisement call demonstrates that the sound energy is distributed among the fundamental frequency (F_0) and higher harmonics (F_{1-10}). Background noise in the tanks was caused by an aquarium chiller which turned on and off periodically and a water pump, which ran continuously. Power spectral density curve of the combined sound from the chiller and pump (pink). Power spectral density curve of the sound emanating from the pump when the chiller was in the off phase (green). The spectrogram in (B) was generated in Raven Pro v1.5 with the following settings, a Hann window with 13606 samples and a 3 dB filter bandwidth of 4.66 Hz, a hop size of 6803 samples with 50% overlap, frequency grid with grid spacing 2.69 Hz and DFT size 16384. For the individual in (B and C), F_0 was close to 90 Hz, with the higher harmonics being integral multiples of F_0.
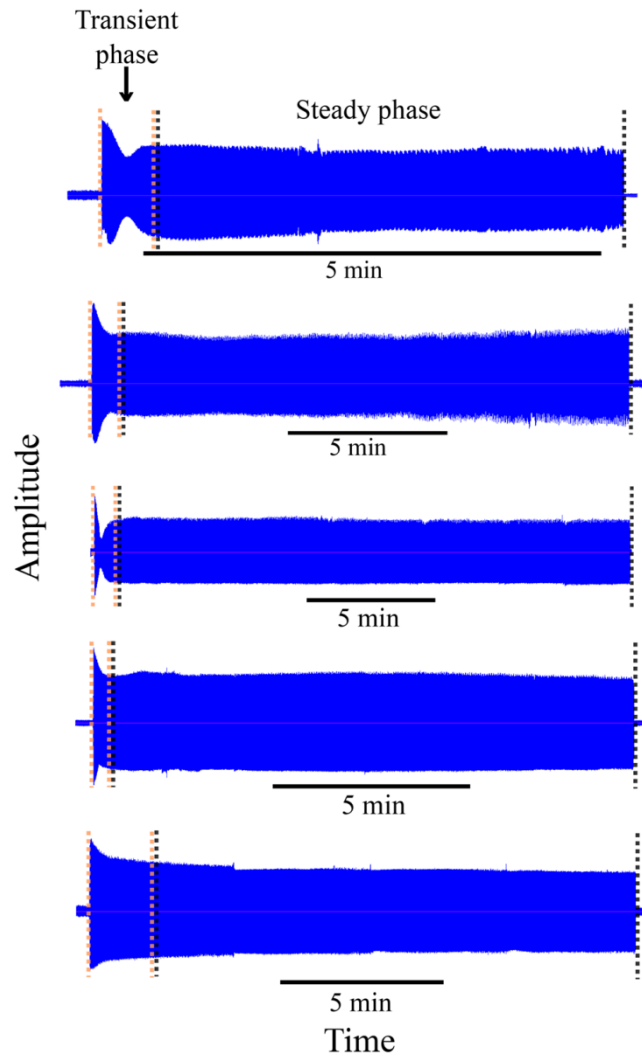
Figure 3. Oscillograms of five representative advertisement calls reveal that the call has an initial transient phase marked by a sharp rise and fall in pressure amplitude, followed by a steady phase where there is little change in pressure amplitude. Pressure amplitude is plotted in voltage units of the Zoom H2 digital recorder used to record the advertisement calls produced by calling type I males.
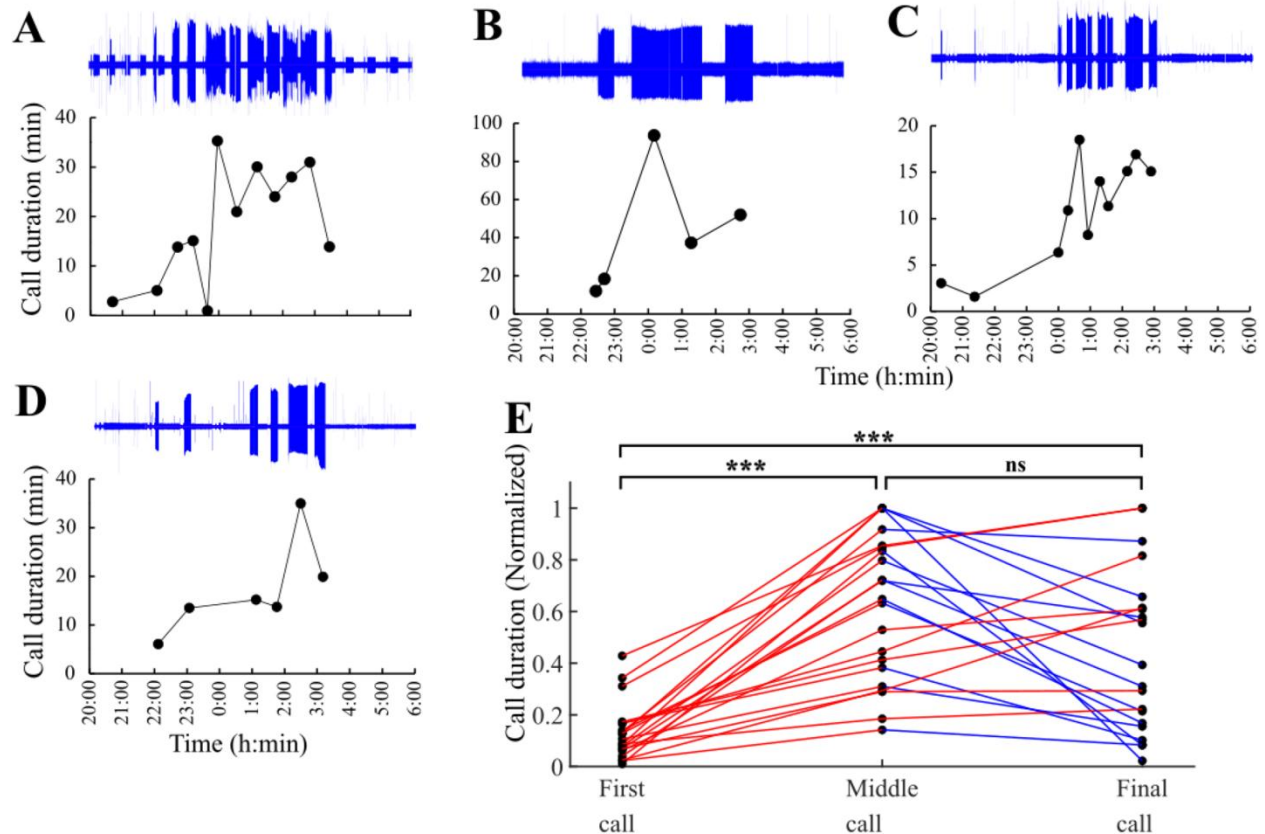
Figure 4. Temporal trends in call duration. (A-D) Oscillogram (Top), duration (Bottom) of the advertisement call as a function of time of the night for 5 representative individuals. Time on the x-axis represents the instantaneous time at the midpoint of each advertisement call. (E) Normalized duration of the first, middle, and final advertisement call produced by the type I male over the course of a single night. Call position (first, middle, or final) had a significant effect on call duration (Friedman test: $\chi^2(2) =$ 26.95, Kendall's $W = 0.58$, $N = 21$, $P < 0.001$). Pairwise comparisons between the first, middle, and final calls were performed using Conover post-hoc tests with Bonferroni corrections ($N = 21$). *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$. Red and blue lines represent increments and decrements, respectively.
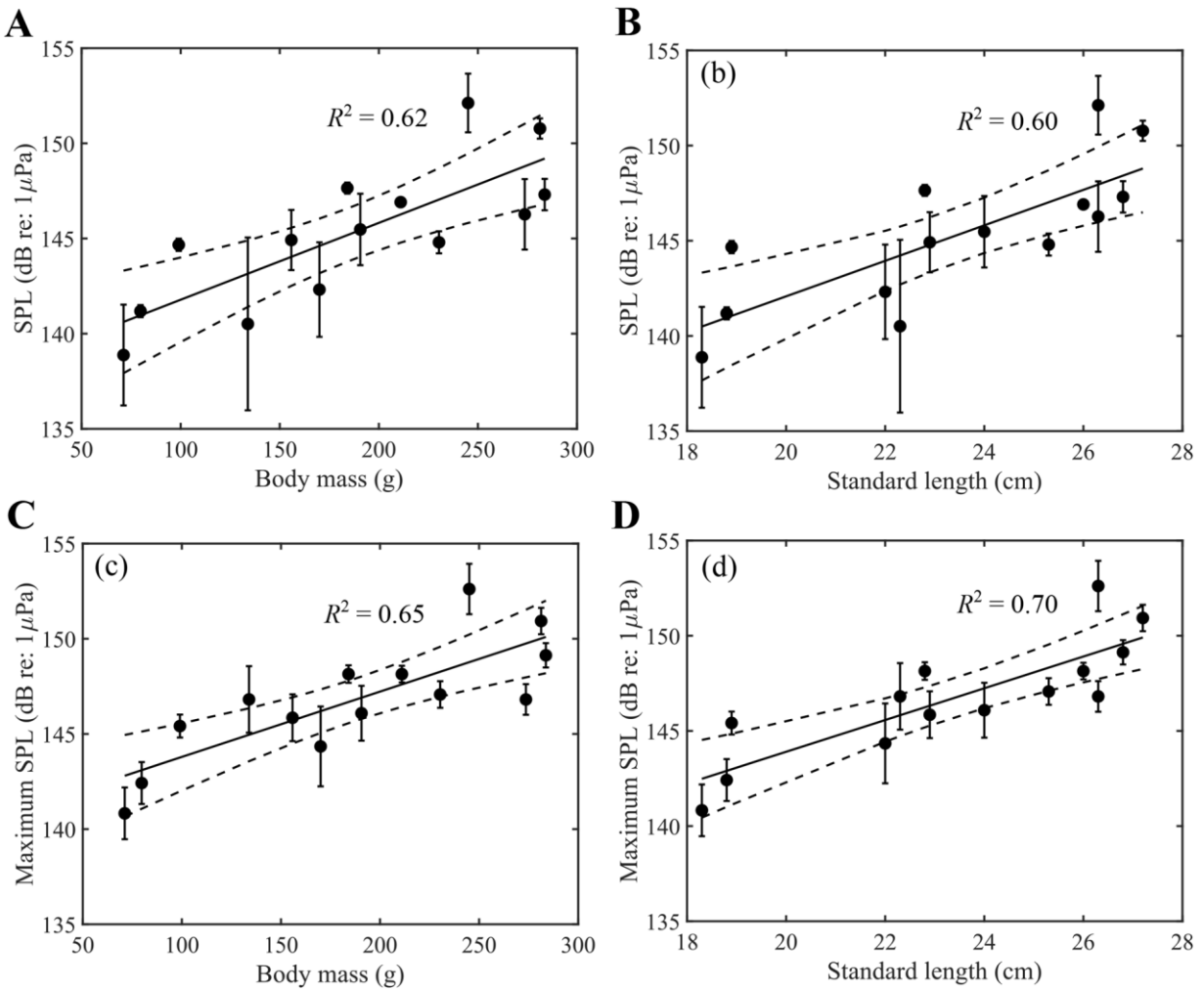
Figure 5. Loudness of the advertisement call reflects body size of advertisement calling type I male midshipman. Sound pressure level (SPL) is highly correlated with (A) body mass ($r_s = 0.81$, $P < 0.001$) and (B) standard length ($r_s = 0.83$, $P < 0.001$). Maximum SPL is also highly correlated with (C) body mass ($r_s = 0.81$, $P < 0.001$) and (D) standard length ($r_s = 0.84$, $P < 0.001$). $N = 14$ for all correlations. Best fit lines, 95% confidence interval bands, and R^2 values of the best fit lines are shown on the plots. Error bars represent mean value + standard deviation in this figure and all subsequent figures.
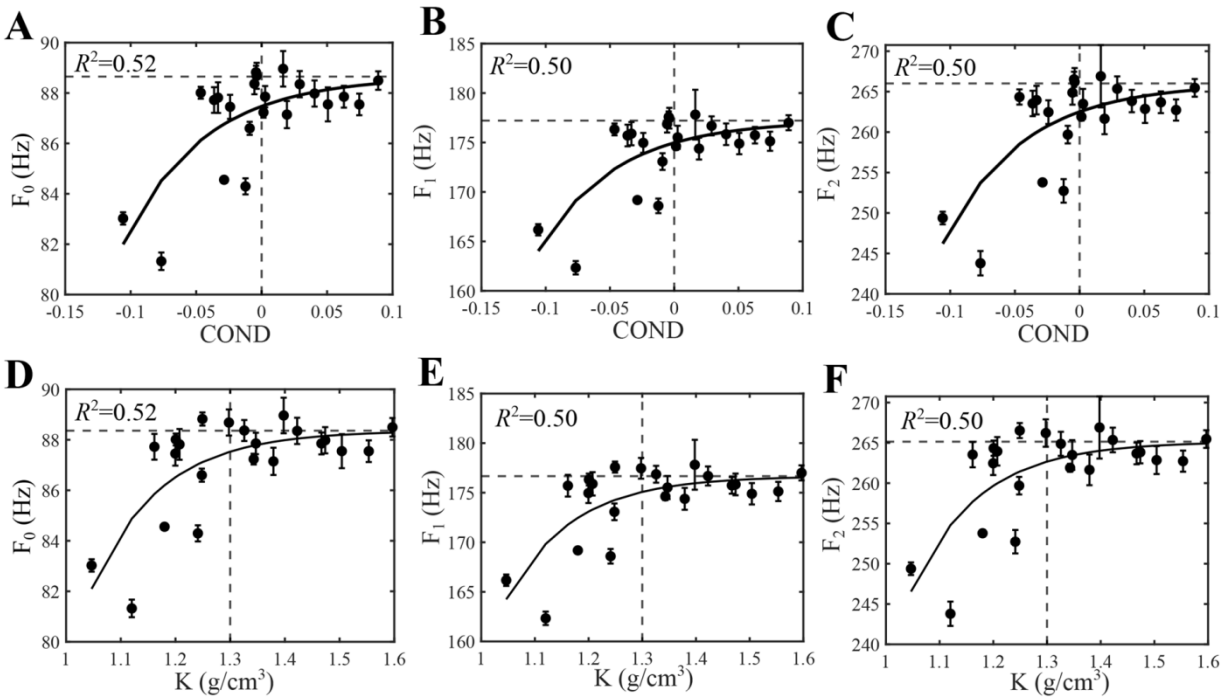
Figure 6. Harmonic frequencies (F_{0-10}) reflect body condition of advertisement calling type I male midshipman. (A-F) Harmonics (F_0-F_2) plotted against two measures of body condition, (A-C) residuals of the regression between log body mass and log standard length (COND), and (D-F) Fulton's condition factor (K) for N = 22 type I males. Black curves represent the best fit asymptotic regression models of the form $y = F_{AS} - (F_{AS} - I)*e^{-kx}$ where y= Harmonic frequency (F_{0-2}), x= Body condition (COND or K), F_{AS} is the asymptotic frequency at higher body condition, I is the intercept, and k represents the rate of exponential decay. R^2 values of the best fit lines are shown on the plots. Dashed horizontal lines represent the asymptotic frequency (F_{AS}) and dashed vertical lines represent the approximate inflection point where harmonic frequency plateaus with increasing body condition (COND or K).

Table 1. Spearman's rank correlation coefficients (r_s) between morphometric parameters and acoustic features of advertisement calling type I male midshipman, namely sound pressure level (SPL), maximum SPL and fundamental frequency (F_0). See METHODS for more details. *, **, and *** represent $P < 0.05$, $P < 0.01$, and $P < 0.001$.

	SPL	Maximum SPL	F_0
Body mass	$r_s = 0.81$*** $N = 14$	$r_s = 0.81$*** $N = 14$	$r_s = 0.28$ $N = 22$
Standard length	$r_s = 0.83$*** $N = 14$	$r_s = 0.84$*** $N = 14$	$r_s = 0.22$ $N = 22$
COND	$r_s = 0.23$ $N = 14$	$r_s = 0.07$ $N = 14$	$r_s = 0.42$ $N = 22$
K	$r_s = 0.36$ $N = 14$	$r_s = 0.25$ $N = 14$	$r_s = 0.48$* $N = 22$
Swim bladder mass	$r_s = 0.75$** $N = 14$	$r_s = 0.85$*** $N = 14$	$r_s = 0.37$ $N = 14$
Sonic muscle mass	$r_s = 0.63$* $N = 14$	$r_s = 0.75$** $N = 14$	$r_s = 0.09$ $N = 22$
Liver mass	$r_s = 0.64$* $N = 14$	$r_s = 0.58$* $N = 14$	$r_s = 0.47$ $N = 14$
Hepatosomatic index	$r_s = -0.24$ $N = 14$	$r_s = -0.41$ $N = 14$	$r_s = 0.29$ $N = 14$
Gonadosomatic index	$r_s = 0.19$ $N = 14$	$r_s = 0.15$ $N = 14$	$r_s = -0.27$ $N = 14$

Table 2. R^2 values of the best fit asymptotic regression models to explain the relationship between harmonic frequencies (F_{0-10}) and two body condition measures (COND and K) of advertisement calling type I male midshipman. The regression model was of the form $y = F_{AS} - (F_{AS} - I)*e^{-kx}$ where y= Harmonic frequency (F_{0-10}), x= Body condition (COND or K), F_{AS} is the asymptotic frequency at higher body condition, I is the intercept, and k represents the rate of exponential decay.

Harmonic frequency	R^2 (COND)	R^2 (K)
F_0	0.52	0.52
F_1	0.50	0.50
F_2	0.50	0.50
F_3	0.50	0.49
F_4	0.49	0.49
F_5	0.50	0.50
F_6	0.50	0.50
F_7	0.49	0.50
F_8	0.50	0.50
F_9	0.49	0.49
F_{10}	0.49	0.49

Table 3. Spearman's rank correlation coefficients (r_s) between morphometric parameters and acoustic features related to type I male calling activity (calling rate, calling effort, and call duration), and energy distribution of the harmonic frequencies in the advertisement call, namely aggregate entropy (H) and harmonic decay rate (b). See MATERIALS AND METHODS for more details.

|	Calling rate	Calling effort	Call duration	H	b
Body mass	$r_s = -0.21$ $N = 22$	$r_s = -0.29$ $N = 22$	$r_s = -0.14$ $N = 22$	$r_s = -0.01$ $N = 22$	$r_s = 0.25$ $N = 22$
Standard length	$r_s = -0.23$ $N = 22$	$r_s = -0.26$ $N = 22$	$r_s = -0.08$ $N = 22$	$r_s = -0.11$ $N = 22$	$r_s = 0.21$ $N = 22$
COND	$r_s = 0.00$ $N = 22$	$r_s = -0.21$ $N = 22$	$r_s = -0.25$ $N = 22$	$r_s = -0.39$ $N = 22$	$r_s = 0.30$ $N = 22$
K	$r_s = -0.04$ $N = 22$	$r_s = -0.25$ $N = 22$	$r_s = -0.28$ $N = 22$	$r_s = -0.38$ $N = 22$	$r_s = 0.33$ $N = 22$
Swim bladder mass	$r_s = -0.24$ $N = 14$	$r_s = -0.18$ $N = 14$	$r_s = 0.19$ $N = 14$	$r_s = -0.10$ $N = 14$	$r_s = 0.26$ $N = 14$
Sonic muscle mass	$r_s = -0.09$ $N = 22$	$r_s = -0.16$ $N = 22$	$r_s = -0.13$ $N = 22$	$r_s = 0.10$ $N = 22$	$r_s = 0.12$ $N = 22$
Liver mass	$r_s = -0.23$ $N = 14$	$r_s = -0.35$ $N = 14$	$r_s = -0.09$ $N = 14$	$r_s = -0.44$ $N = 14$	$r_s = 0.51$ $N = 14$
Hepatosomatic index	$r_s = 0.02$ $N = 14$	$r_s = -0.13$ $N = 14$	$r_s = 0.10$ $N = 14$	$r_s = -0.16$ $N = 14$	$r_s = 0.24$ $N = 14$
Gonadosomatic index	$r_s = -0.04$ $N = 22$	$r_s = -0.13$ $N = 22$	$r_s = 0.06$ $N = 22$	$r_s = 0.31$ $N = 22$	$r_s = -0.42$ $N = 22$
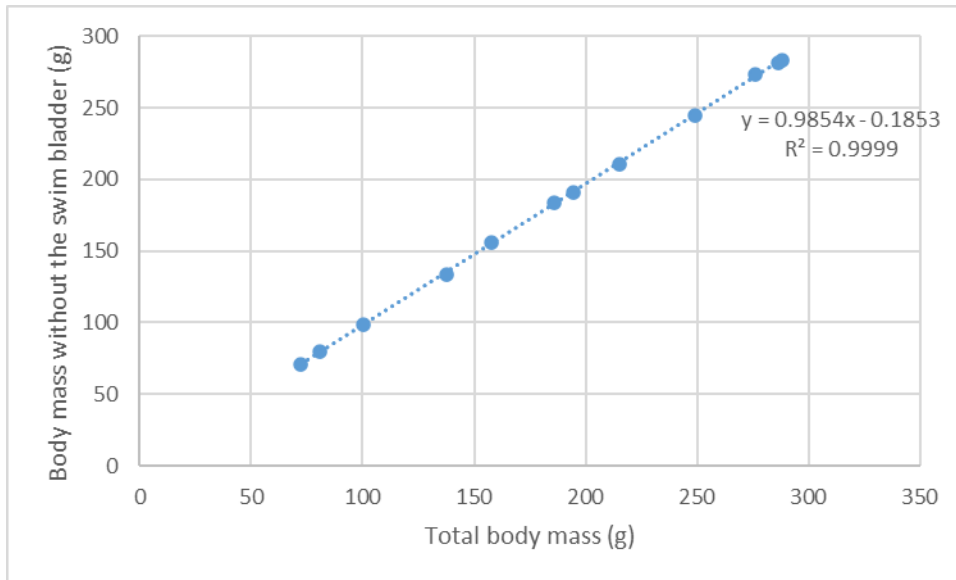
Fig. S1. Body mass (without the swim bladder) versus total body mass of advertisement calling type I males.
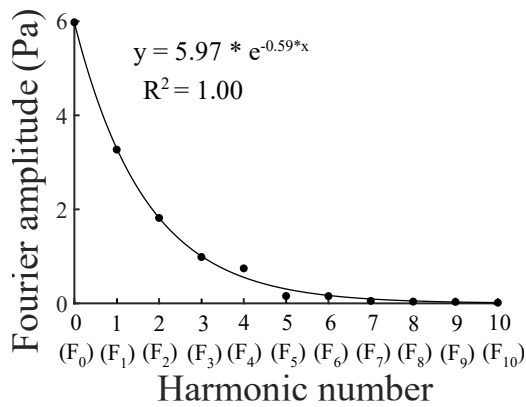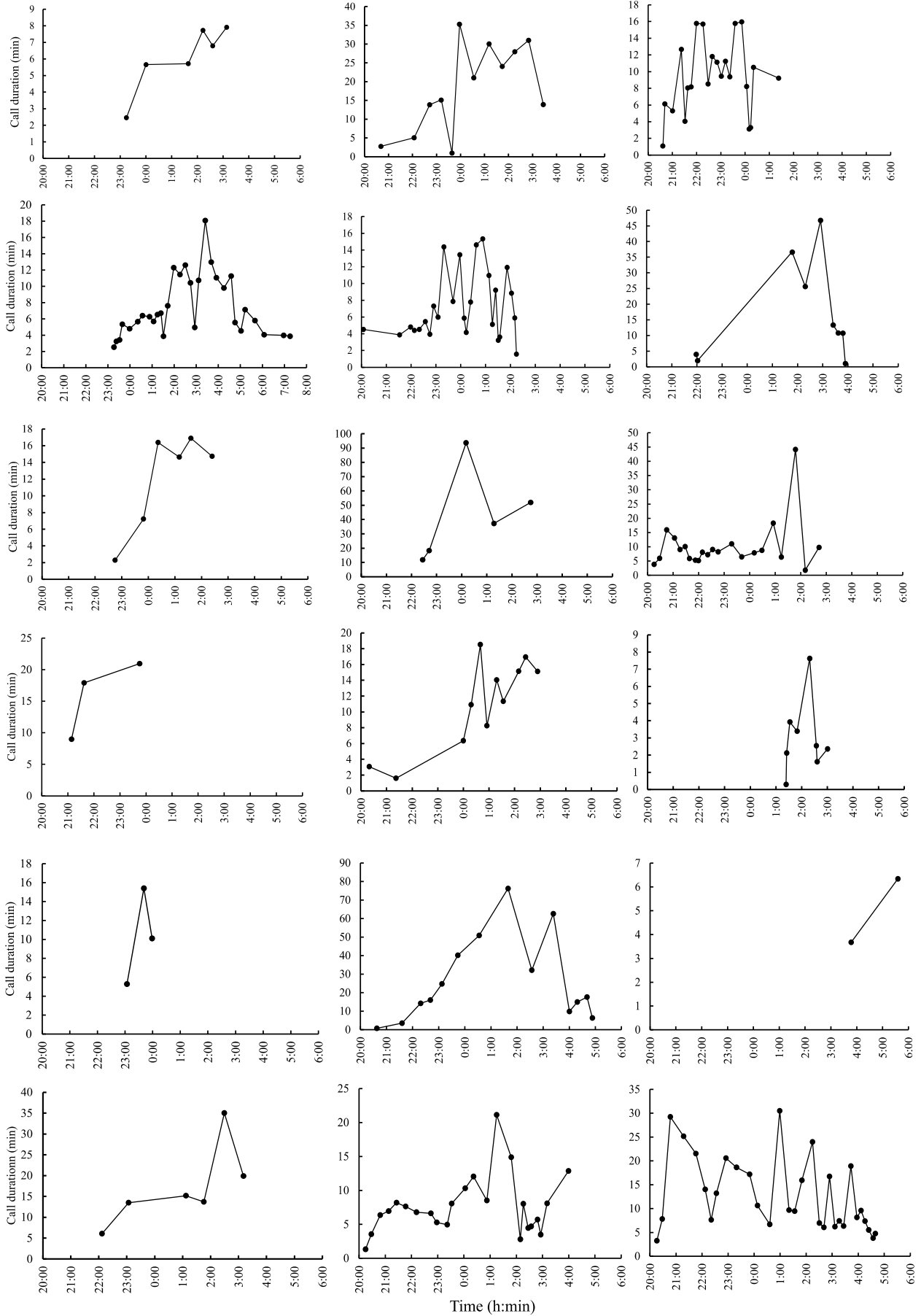

Fig. S2. Fourier amplitude plotted as a function of harmonic number for a single representative 1 minute call segment. For this specific advertisement call, an exponential decay function of the form $y = a * e^{-bx}$ fit the data almost perfectly, $R^2 \approx 1$, where a= 5.97 and b=0.59. b represents the harmonic decay rate.
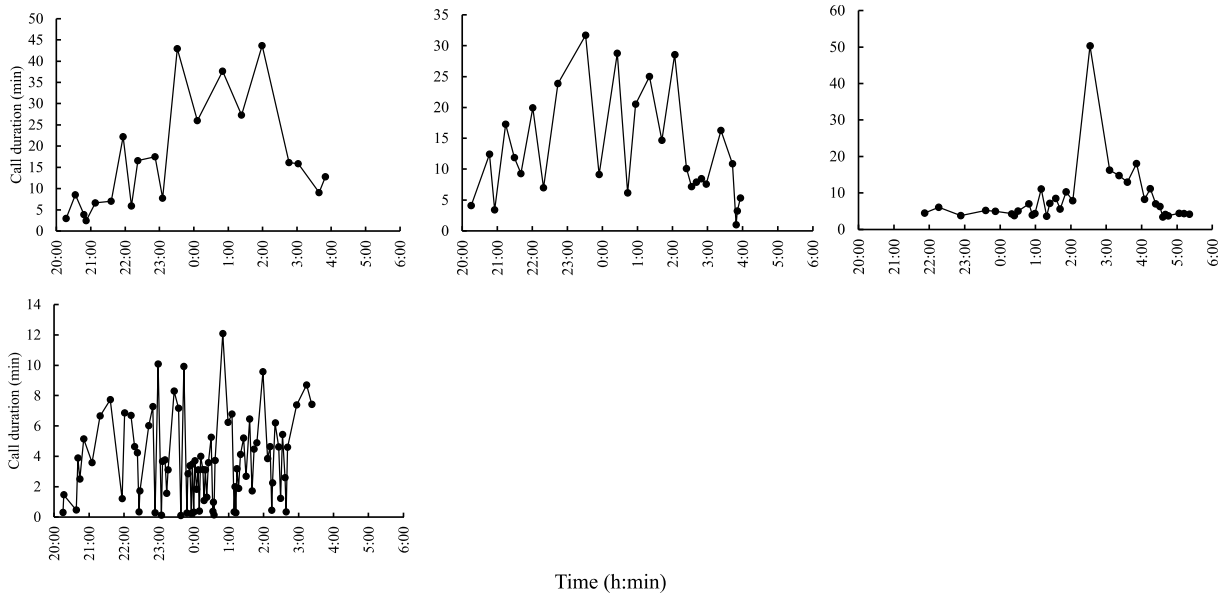
Fig. S3. Call duration versus time for all 22 individuals.