Male antiphonal calls and phonotaxis evoked by female courtship calls in the large odorous frog (Odorrana graminea)

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Systematic Review

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Abstract

Acoustic communication plays a vital role in frog reproduction. In most anuran species, long-distance sound communication is one-way- from males to females; during the reproductive season males produce species-specific advertisement calls to attract gravid females, and females are generally silent but perform phonotactic movements that lead to amplexus. One exception is the concave-eared torrent frog (*Odorrana tormota*). In this species, females produce courtship calls that elicit antiphonal vocalizations by males, followed by precise phonotactic movements. The large odorous frog *O. graminea* in southern China, is subject to the same environmental constraints as *O. tormota* with which it is sympatric; it is unclear whether their sound communication is one-way or bidirectional. Here we provide the first data on female *O. graminea* vocalizations and their functions. Using playbacks of female calls, we conducted acoustic behavioural experiments in the laboratory in response to which males emitted single- or multi-note antiphonal calls with a varying fundamental frequency. Moreover, they were attracted to female call playbacks, exhibiting precise phonotaxis. The female courtship call □ male response interaction thus forms a duet between partners of a receptive pair. These results demonstrate that this unique communication system likely reflects an adaptation to an environment in which short-distance communication is at a premium given the high levels of ambient noise.

Introduction

Acoustic communication plays a vital role in frog reproduction. During the reproductive season, males of most frog species emit species-specific advertisement calls to attract gravid females; females without vocal sacs are generally silent but exhibit positive phonotaxis that leads to amplexus (Endler 1992; Bush 1997; Tobias et al. 1998; Ryan 2001; Narins et al. 2007). One exception is the concave-eared torrent frog (*Odorrana tormota*). In response to males' calls, females of *O. tormota* produce broadband courtship calls with energy extending into the ultrasonic range; males can detect ultrasound, thus avoiding masking by the intense, predominantly low-frequency ambient noise. Female calls typically elicit one precisely timed antiphonal vocalization from males, followed by male phonotaxis (Feng et al. 2006; Shen et al. 2008; Shen 2018; Shen and Xu 2019). The large odorous frog (*Odorrana graminea*), a sympatric species, is subject to the same environmental constraints as *O. tormota*. Previous results show that males produce diverse broadband signals most of which contain ultrasonic harmonics (Narins et al. 2004; Shen et al. 2011a) and have the ability to detect ultrasound (up to 24 kHz) (Arch et al. 2011; Feng et al. 2006; Shen et al. 2008; Liu et al. 2014). However, it is unclear whether female *O. graminea* are silent or produce courtship calls. The present study demonstrates that female *O. graminea* can produce courtship calls when oviposition is imminent. The female courtship calls are functionally a kind of fertility advertisement that evokes a male's immediate antiphonal responses and phonotactic movements, finally leading to amplexus. Thus, we believe that there is a bidirectional sound communication system between gravid females and receptive males in *O. graminea*. These results should stimulate further studies to provide new insights into the mechanisms underlying bidirectional acoustic communication in anuran amphibians.

Materials And Methods

Animal preparation

The large odorous frog (*O. graminea* Boulenger) is an arboreal, nocturnal species living near noisy streams and waterfalls in mountainous areas (elevation 450-1200 m) of southern China. These frogs are sexually dimorphic with adult males having a snout-vent length of ca. 48 mm and females are much larger having an S-V length of ca. 91 mm (Fei et al. 2009). At the peak of the breeding season, we recorded the calls of *O. graminea* between 19:30h and 21:30h on 13 to 29 May 2007 along the Tau Hua Creek in Huangshan Hot Springs, Anhui, China (30.101° N, 118.173° E) at an elevation of ~640 m. The acoustic behavioural experiments were conducted from 30 July to 8 August 2010 and from 5 to 26 July 2011 in a laboratory in Huangshan Hot Springs with an ambient temperature and humidity of ~22°C and ~60%, respectively.

Seven gravid females and sixteen males of *O. graminea* were collected from the Tau Hua Creek on rainy nights. In the field, an amplexed pair were uncoupled by hand, then kept separately in a visually opaque but acoustically transparent plastic cages in a quiet, darkened room about 1 km away from their natural habitat.

Frog call recordings

About 30 minutes after capture, indoor recordings were carried out overnight prior to oviposition and hormonal level reduction. In a quiet, darkened, anechoic room under dim infrared illumination, males were placed in an indoor gauze-fenced arena (1.5 × 1.3 x 0.86 m), where a captive female in her cage was separated from a male in his cage by about 3 m. In this way, most types of calls, including female natural calls, male elicited antiphonal calls and staccato calls, were recorded with a ¼-inch wide-band condenser microphone and a preamplifier (models 40BE and 26CB, respectively, G.R.A.S. Sound & Vibration, Holte, Denmark; frequency response, 4 Hz~100 kHz, ±3 dB) placed above the cage, and a digital audio recorder (Sound Devices model 722, Sound Devices, Reedsburg, WI, USA) with a sampling rate of 96 kHz and 16-bit resolution. The sound pressure level (dB SPL) was compared with the output of a calibrator (Bruel and Kjaer 4231) that produces a 1-kHz tone at 94 dB SPL.

Laboratory phonotaxis studies

Laboratory phonotaxis experiments were conducted in an anechoic room at a temperature of 22 ± 2°C and humidity of ~60%. The room measured 3 m long, 2.5 m wide, and 2.5 m high, with 10-cm thick acoustic foam on all surfaces for sound absorption. Prior to the start of the experiment, three males were freshly captured and kept individually in small plastic cages each night. A rectangular arena measuring 2.0 m by 1.5 m was located within the room. During the phonotaxis experiments, each male frog was placed on the floor in the center of the short side of the arena (that is, the release site) under a removable glass cover (C, inside diameter 8.5 cm; see Fig. 3). The loudspeaker (frequency range: 1~30 kHz; Fostex FE87E) was placed 1.2 m away on the floor opposite to the release site, broadcasting female courtship calls (abbr. FCC) as the playback stimuli presented at one stimulus per 60 s and at an intensity of ~80 dB SPL, measured at the center of the arena. The general experimental protocol followed that described by Shen (2008). Single note FCC or a 'two-note' call (FCC1 and FCC2) exemplars were selected for testing each captive male.

The animal's movements were monitored and recorded under infrared illumination by an infrared video camera (Sony DCR-TRV30E or HDR-SR7). All sound recordings from the field and the lab were transferred to the computer as WAV files, analyzed (fast Fourier transform, 1,024 points), and displayed using SELENA software, a custom-designed program (S. Andrzheevski, St. Petersburg) (Narins et al. 2004; Feng et al. 2006; Shen et al. 2008), and PRAAT (Boersma and Weenick 2008) programs (Feng et al. 2009). At the conclusion of each experiment, all animals were released back to their native sites along the creek.

Statistics

The trajectories for each responsive male frog in the phonotactic experiments were illustrated based on the video recordings. Two parameters were measured: the jump distance (D, in cm) and azimuthal jump angle (α, in degrees). The azimuthal angle of each long distance jump was calculated using the formula $\alpha = \arcsin d / D$, where d is the shortest distance from the male's present position to the straight line between the male's initial position and the center of the loudspeaker.

Results

Female natural call in the laboratory

Prior to ovulation at night, captive gravid females (*O. graminea*) in an indoor arena produce simple calls repeatedly about once every 10 minutes. The female calls immediately stimulate male vocalizations. The audio and video recordings clearly show that the female's natural calls induce receptive males' antiphonal responses followed by phonotactic movements, finally even resulting in amplexus. Therefore, such a call has been shown to be a highly attractive stimulus, called as 'female courtship call' (abbr. FCC). The FCC is a single note, or sometimes a 'two-note' call, of shallow frequency-modulated stacks. The fundamental harmonic sweeps from 4.47 ± 0.57 kHz downward to 2.68 ± 0.61 kHz, across the duration (26.5 ± 8.3 ms, n = 82), with an intensity 85 dB SPL measured at a distance of 50 cm above the female frog's head, much above the ambient noise level at the calling site, that averages from 58 to more than 70 dB SPL in the frequency range between 50 and 4000 Hz. As shown in Fig. 1, a single-note FCC has several harmonics, the frequencies of the fundamental harmonic (F_0 or F_1) to fifth harmonic (F_5) marked by the red vertical line were 5.2 kHz, 10.4 kHz, 15.6 kHz, 20.8 kHz, and 26.0 kHz, respectively, and the call duration was ca. 40 ms with energy extending into ultrasonic range (up to 36 kHz). The 'two-note' call has approximately the same spectrum as a single note FCC, and the second note (FCC2) has a shorter duration (the difference about 8 ms), is less intense (the difference about 5 dB), with an inter-note interval between FCC1 and FCC2 of 255 ± 47 ms (n = 14).

Male antiphonal calls in the laboratory

When a male frog in captivity in a quiet darkened room was separated by the black cotton gauze mesh with a gravid female frog in another small chamber, the male was often induced to vocalize by the female courtship call. Six examples of immediately evoked antiphonal responses (abbr. AR) are shown in Fig. 2. The latencies to the male's first AR were 0.2 s, 0.35 s, 2.7 s, 1.5 s, 1.6 s, and 1.2 s, respectively, measured from the onset of the FCC or the first note of female call (FCC1).

The AR was generally a short upward-downward tonal call with a duration of about 60-85 ms (e.g. Fig. 2a, b), exhibiting a number of harmonics and different F_0, although recorded from the same male. The mean F_0 of a single AR note was 4.47 kHz, maximum F_0 4.75 kHz, minimum F_0 4.15 kHz, as shown in Fig. 2a, and the frequency of the harmonics marked by the red vertical line was 4.9 kHz, 9.8 kHz, 14.7 kHz and 19.6 kHz, respectively. In Fig. 2b, the mean F_0 of a single AR was 5.66 kHz, maximum F_0 6.26 kHz, minimum F_0 4.89 kHz, and the frequency of these harmonics marked by the red vertical line was 6.3 kHz, 12.6 kHz, 18.9 kHz and 25.2 kHz, respectively.

Sometimes the ARs were short frequency-modulated (FM) calls, frequently followed by a multi-note long call with a duration of 600~800 ms, including 4-5 separate notes, as shown in Fig. 2c-f. The analysis results show that the mean F_0 of an individual AR note varied. For example, the ARs were often quite complex, when evoked by a single FCC (Fig. 2c, f) or a 'two-note' call (FCC1 and FCC2), as in Fig. 2d, e.

The mean F_0 of eight elicited AR notes in Fig. 2c was 3.0 kHz, 2.6 kHz, 0.6 kHz, 4.1 kHz, 4.7 kHz, 5.9 kHz, 4.3 kHz and 4.5 kHz, respectively. In Fig. 2d, the mean F_0 for seven elicited AR notes was 2.6 kHz, 2.6 kHz, 3.6 kHz, 3.9 kHz, 5.0 kHz, 4.9 kHz and 5.8 kHz, respectively. In Fig. 2e, the mean F_0 for five elicited AR notes was 2.6 kHz, 2.7 kHz, 3.1 kHz, 4.0 kHz, and 3.5 kHz, respectively. In Fig. 2f, the mean F_0 for eight elicited AR notes was 2.2 kHz, 1.8 kHz, 2.0 kHz, 2.3 kHz, 2.6 kHz, 3.3 kHz, 11.6 kHz and 12.7 kHz, respectively. Moreover, the last long call in Fig. 2c-f was like a singing tenor- characterized by pronounced and varying frequency modulation patterns, and occurrence of nonlinear phenomena (i.e., frequency jumps, subharmonics, and deterministic chaos), and having energy in the ultrasonic range up to ca. 42 kHz. As far as we know, only males of *O. graminea* have such a complicated and varied antiphonal response. Its functional significance may be an important trait that transmits a male's fitness to the female. These ARs were rare in other anuran species (Ryan 1985; Narins et al. 2000).

Male phonotaxis in the laboratory

We found that in a quiet, dark indoor arena, newly captured males were most responsive during the phonotaxis experiments. In response to the FCC stimuli at a playback level of 85 dB SPL, in addition to vocal responses evoked, males rapidly and accurately localized the loudspeaker. The representative phonotactic trajectories elicited from the males by the FCC playback are illustrated in Fig. 3. The FCC playback resulted in the attraction of receptive males to the speaker. Analysis of the speaker-derived phonotactic paths indicated that males of *O. graminea* are mainly characterized by fast response with a minimum latency of less than 1 s, a long jump with a maximum distance more than 1 meter, and extraordinary localization acuity. Upon hearing the FCC playback, a receptive male immediately turned his body towards the speaker and made a long jump (range: ca. 60-100 cm) toward the speaker. Most males would go directly to or near the loudspeaker, sometimes touching the foam on the wall above the loudspeaker, and falling. A small number of males jumped a short distance (ca. 30 cm) and then quickly crawled toward the playback speaker. When the male frog heard the second or third acoustic stimulus (FCC playback), it would immediately orient itself, adjust its trajectory, take a big leap, and make contact with the speaker, possibly leading to amplexus if the sound source were a live gravid female frog. Therefore, we believe that the FCC is a salient stimulus and has a significant attraction to males.

The precision of the long-distance jumps (more than 30 cm) of receptive male *O. graminea* was remarkable, with an average azimuthal error of jumps just 0.7° ± 0.8° (n = 14), even smaller than that in male concave-eared frogs (Shen et al. 2008). This accuracy suggests that large odorous frogs have the capacity to localize their mates in noisy, dark habitats.

Discussion

The large odorous frog *O. graminea* is an arboreal, nocturnal species which inhabits the vegetation along noisy streams, rendering acoustic communication challenging; one solution is to advertise conspicuously. Males of *O. graminea* have been shown to produce various calls with spectral energy extending into the ultrasonic range to avoid masking from the broadband ambient noise and to detect ultrasound up to 24 kHz (Feng et al. 2002, 2006; Narins et al. 2004; Shen et al. 2011a; Liu et al. 2014; Shen 2018). In this study, we found that like *O. tormota*, female gravid frogs of *O. graminea* also emit courtship advertisement calls when oviposition is imminent. Functionally, female courtship advertisement calls elicit both male vocalization and approach. Therefore, the female call–male answer interaction forms a duet between partners of a receptive pair. In addition, we found that the playback of the male advertisement call can induce accurate phonotactic behavior of female frogs as well (not shown here), and the main characteristics are the short distance jump and rapid crawling due to their larger weight. We believe that the bidirectional sound communication indeed plays an important role in both *O. graminea* and *O. tormota* reproduction.

The present results further show that in comparison with usual advertisement calls in *O. graminea* (Shen et al. 2011a), receptive males emit distinct antiphonal calls of longer duration, in particular, with drastic changes in pitch to overcome the masking effects of ambient noise, to increase the salience of the communication signal, to have more attraction of gravid females, to facilitate amplexus and to ensure successful reproduction in the field.

In the laboratory, the fact that female call stimuli emitted from a point source (speaker) reliably elicit male's first antiphonal response with unequal latency ranging from 0.2 s to 2.7 s (see Fig. 2) suggests that recognition, rather than localization, is likely the primary problem. We observed that when the second or third stimulus was presented to the male frog during his transit toward the loudspeaker, the frog suddenly adjusted its body orientation, and then made a precise jump toward the sound source. This suggests that for the male frog the recognition comes first, and the localization is secondary.

The present studies indicate that males of *O. graminea* can localize a calling female or a loudspeaker with an extraordinary acuity of less than 1°, despite their small head size (interaural distance less than 2 cm). However, most amphibians are less well endowed, generally showing an acuity of about 16–23° (Christensen-Dalsgaard 2005), as they locate a sound source based on low-frequency perception. In contrast, ultrasonic males of *O. tormota* have the capacity to perceive higher-frequency sounds as an adaptation to their noisy habitats, which may underlie their hyperacute sound localization. Additional mechanisms that underlie localization hyperacuity in *O. graminea* remain to be studied.

It is worth mentioning that we have conducted field investigations along the Shunyan Creek (Hongyangou) Hejiang, Sichuan Province, China (28.640°N, 106.138°E) on 30 May–10 June 2009 and on 28 May–7 June 2010, respectively, at elevations >701 m. Fig. 4 shows that the main members of the research team investigating the habitat of the large odorous frogs (*O. graminea*) along the Shunyan Creek in June 2009. We recorded sporadic calls of male frogs in this very noisy habitat. No gravid female frogs were found. When playing back the female calls recorded from Huangshan, Anhui Province, China, males' antiphonal calls and phonotactic movements were not observed in the habitat along the Shunyan Creek. We believe that the main reason for this was that the peak breeding season of the large odorous frogs may be displaced by three weeks in Hejiang, Sichuan, China compared to that in Huangshan, China.

Declarations

Acknowledgments

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Author Contributions

J.X.S. was responsible for the research. All authors (except P.M.N.) conducted the frog vocalization and behavioural experiments, J.X.S. and Z.M.X. analyzed the behavioural data and video recordings, and J.X.S. analyzed the acoustic data. All authors contributed to writing the manuscript.

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Figures

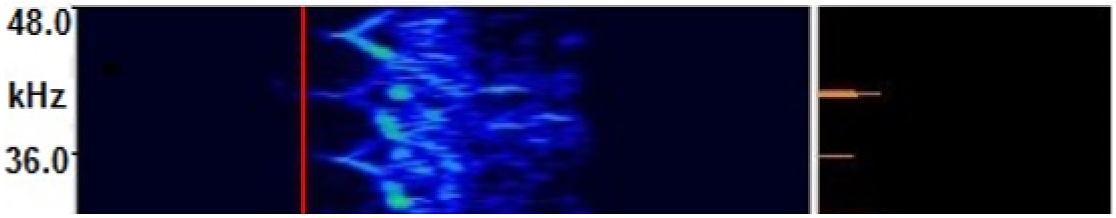

Figure 1

A single-note female courtship call of the large odorous frog *Odorrana graminea* in Anhui Province, China. It is comprised of several harmonics, extracted from the audio recordings, showing the oscillogram (*bottom*), spectrogram (*top left*) and instantaneous amplitude spectrum (*right*) (taken at the time indicated by the red line).

Figure 2

a-f. Either the female courtship calls (abbr. FCC) or playbacks of the FCC evoke antiphonal responses (abbr. AR) from males of *O. graminea*, showing the oscillogram (*bottom*), spectrogram (*top left*) and instantaneous amplitude spectrum (*top right*) (taken at indicated red lines). To clearly display the calls, the dotted lines on the oscillogram indicated the silent segments that were deleted from the base line (the numbers in sec). **a-b, f** The FCC playback made from the audio recording evoked male ARs. **c-e** Female calls and male ARs were extracted from the video-recordings.

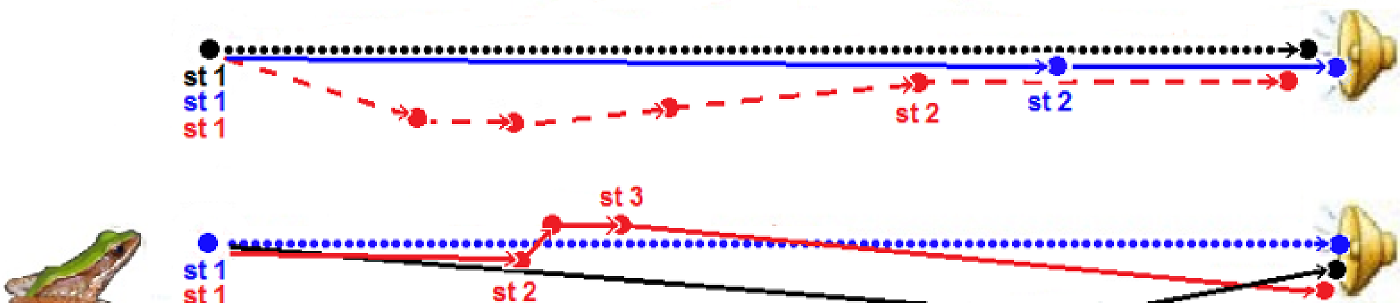

Figure 3

Phonotactic movements of receptive males evoked by female courtship calls in the large odorous frog *O. graminea*. The nine representative paths were based on the video-recording data from 13 to 23 July, 2011. Different colors indicate different experimental male frog individuals. Filled circles: the start and landing site of a jump. Arrows: a jump's direction towards a loudspeaker, broadcasting the FCC stimulus. Inset: an image of a male of *O. graminea*. st1, st2, st3 indicate the male's position at the time of the first, second or third stimulus (FCC) presented by the loudspeaker.

Figure 4

The main members of the research team investigating the habitat of the large odorous frogs (*O. graminea*) along the Shunyan Creek, Hejiang, Sichuan, China in June 2009. From *left* to *right*: Jun-Xian Shen, Albert S. Feng (University of Illinois at Urbana-Champaign, USA.), Peter M. Narins, Zhi-Min Xu, and Jian-Ping Jiang (Chengdu Institute of Biology, Chinese Academy of Sciences). Photo: J-X Shen