Ultrasonic frogs show extraordinary sex differences in auditory frequency sensitivity

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Acoustic communication plays an important role in the reproductive behavior of anurans. Males of concave-eared torrent frog (*Odorrana tormota*) have ultrasonic communication capacity [1, 2], but it is unknown whether females communicate with ultrasound. Here we show that *O. tormota* exhibits great sex differences in the auditory frequency sensitivity. Acoustic playback experiments demonstrated that the male's advertisement calls evoke gravid females' positive phonotaxis and vocal responses, whereas ultrasonic components of the male's calls (frequencies above 20 kHz) do not elicit female phonotaxis or vocalization. The behavioral study was complemented by electrophysiological recordings from the auditory midbrain and by laser Doppler vibrometer measurements of the tympanic membrane's response to acoustic stimuli. These measurements revealed that females have an upper frequency limit up to 16 kHz (threshold 107 dB SPL) and no ultrasound sensitivity, unlike males which have an upper frequency limit of up to 35 kHz (87 dB SPL). Single units in the female auditory midbrain have the best excitatory frequencies (*BEFs*) peaked around 5 kHz, corresponding to the fundamental frequency (F_0) of male's most calls, whereas the male auditory midbrain units have *BEF*s mostly above 8 kHz, largely consistent with the F_0 of female courtship calls. Females have a frequency sensitive bandwidth (10 dB above threshold) ranged from 2 to 6 kHz, narrower than that males have (5-20 kHz). The velocity amplitude of the tympanic membranes peaked around 5 kHz in females, whereas 7 kHz in males. The results suggest that the frog species *O. tormota* is an example of a vertebrate, which demonstrates well phonotaxis and

extraordinary sex differences in hearing.

The concave-eared torrent frogs *Odorrana tormota* emit high-pitched calls with harmonic energy extending into the ultrasonic range during the reproductive season [1-3]. Males produce a variety of calls (e.g., long and short call, and staccato call) [2-4] and are able to detect ultrasonic components of calls, due in part to having ear canal and ultra-thin tympanic membranes [1], whereas females produced distinct calls in the spectral and temporal features [2].

Acoustic playback experiments were carried out immediately after female frogs were captured at night. The three basic call-types of males' vocalizations (long call, short call and staccato call) [4] and their filtered ultrasonic components were used as acoustic stimuli and played back through the Play unit at 90 dB SPL (sound pressure level) as described [5]. The playbacks of whole spectra of long or short call frequently evoked gravid females' movements (e.g., jump and/or creep). A gravid female frog promptly oriented her body toward the loudspeakers and then hopped onto them by one or more jumps per trial (jump range: ~40-100 cm) with a localization error of 1.3 $^{\circ} \pm 1.7^{\circ}$ (total 60 jumps, 12 females; Fig. 1a) under the long call stimulation (53 of 60, 88.3 %; Supplementary Movie SM-1a, SM-1b), or short call (4 of 60, 6.7 %; Supplementary Movie SM-2) and staccato call (3 of 60, 5 %). When staccato call stimuli (a dominant frequency of ~4 kHz) were presented, gravid females crept rapidly to the loudspeakers with latency of 4.1 ± 3.1 s.

In particular, the playback of whole spectra of the male long call, short call or staccato call elicited the evoked vocal responses (EVRs) from gravid females, starting

immediately after the stimulus onset (delay time, mean $\pm$ SD, 7.16 $\pm$ 2.43 s; a total of 34 EVRs; Supplementary Movie SM-3a, SM-3b). The female antiphonal calls characteristic of a frequency-modulation pip with multiple harmonics (Fig. 1b) have the F_0 of 8.13 $\pm$ 0.97 kHz ($n = 34$), higher than the F_0 of the male long call (about 6.0 kHz) (Fig. 1c). The EVRs are of the most common biological role for mate location, because female courtship calls can in turn induce receptive males' antiphonal calls and rapid and hyperacute phonotactic approaches, as demonstrated in a previous study [2]. All actions facilitate the bond between the sexes amidst low-frequency ambient noise produced by nearby streams and waterfalls.

However, neither EVR nor movement could be evoked from examined twelve females by playbacks of filtered ultrasonic components of the male calls. It indicates that females of *O*. *tormota* are insensitive to ultrasound, although males communicate well with ultrasound during the male-male territorial interactions [1, 2].

To physiologically validate that females of *O*. *tormota* have no ultrasonic sensitivity, auditory evoked potentials (AEPs) from the torus semicircularis (TS; the frog's auditory midbrain) were extracellularly recorded in response to tone bursts (frequency range 1-35 kHz). The female AEPs were consistently observed in response to tone bursts at frequency ranged from 1 kHz to 14 kHz and constant intensity of 90 dB SPL, and occasionally at 15 kHz, 103 dB SPL (Fig. 2a). No AEP was detectable from seventeen examined females using the stimuli at frequencies above 16 kHz and intensity of 110 dB SPL. The peak-to-peak AEP amplitudes measured at 90 dB SPL were normalized and plotted with a log-frequency axis in Fig. 2b (mean $\pm$ SE; total

AEP's amplitudes $n_f = 95$ for females; $n_m = 61$ for males), which showed evidently sex differences with a single prominent peak at 5 kHz in females (in *red*) versus 10 kHz in males (in *blue*). The frequency width at half maximum of the averaged AEP amplitude versus frequency curves (in *dashed line*) was ranged from about 3 kHz to 22 kHz for males versus from about 1 kHz to 10 kHz for females. Fig. 2c delineated the averaged thresholds versus frequency relationships in the female and male *O. tormota* respectively, showing the characteristic frequency (*CF*), or frequency of minimum threshold, at 5 kHz in females (46.9 ± 0.9 dB SPL; total AEPs $n_f = 66$ for females; in *red*) and at 10 kHz in males (54.8 ± 0.87 dB SPL; total AEPs $n_m = 41$ for males; in *blue*). The maximum threshold of hearing (107 dB SPL) was found at 16 kHz in females, whereas 87 dB SPL at 35 kHz in males. Thus, 16 kHz and 35 kHz were regarded as the audible upper frequency limit of females and males, respectively. Moreover, the auditory sensitive frequency bandwidth (at 10 dB above threshold at *CF*) ranged from 2 to 6 kHz in females and from 5 to 20 kHz in males (Fig. 2c; in *dashed line*). The male is l0 dB less sensitive than the female. The Q_{10}-dB (*CF*/bandwidth at 10 dB above threshold) was calculated of 1.25 for females and 0.67 for males, indicating different sharpness of frequency tuning.

A total of 218 single units were recorded from the TS of the species (11 females, 30 males) to determine the best excitatory frequency (*BEF*) at the lowest intensity. The distribution of single units' *BEF*s in females significantly differed from that in males as illustrated in Fig. 3a. For females, most units (82.4 %, 89/108 units) had the *BEF*s between 4 and 6 kHz with a peak at 5 kHz (38.9 %, 42/108), and no unit of the

BEF >10 kHz was found, whereas approximate half the units in males (44.5 %, 49/110) had the *BEF*s between 8 and 10 kHz, 16.4 % (18/110) between 14 and 16 kHz, and 2.7 % (3/110) at 20 kHz. Thus, electrophysiological data demonstrate evident sex differences in auditory filters of the concave-eared torrent frog *O. tormota*, in comparison with two species of frogs *Elentherodactylus coqui* [6] and *Hyla ebraccata* [7].

The tympanic membrane's vibration velocity amplitude spectrum of female *O. tormota* was shown in Fig. 3b (5 ears of three females). The laser Doppler vibrometer measurements revealed a spectral range, spanning from 1 kHz to 10 kHz and peak sensitivity at 5 kHz. Above 10 kHz, the velocity amplitude dropped off about 30-40 dB, largely matching to the AEP amplitude vs frequency and threshold vs frequency relationships observed in females (Fig. 2b and c, in *red*). In contrast to females, laser Doppler vibrometry revealed a wideband spectral range from 2 kHz to ~35 kHz and a peak at about 7 kHz in males [8].

In conclusion, the concave-eared torrent frogs *O. tormota* perform acoustically well male-female communication indoors and in field, although the sex differences in the auditory frequency sensitivity are remarkable. The present study found that the auditory sensitive frequency in one sex correlates well with the F_0 of calls in opposite sex in *O. tormota*. The F_0 of males' antiphonal calls ranged from about 5 to 7 kHz [2], corresponding to the best frequency range (4-6 kHz) of the majority of auditory TS neurons in females (Fig. 2c). And the *BEF* of the male most TS units ranged from about 6 to 10 kHz is comparable to the F_0 of the female calls (about 7.5-9.2 kHz) [2]. The higher-frequency-sensitive TS neurons in males are able to detect and tune to the

female's courtship call, especially to the second harmonic (frequency range from about 15 to 20 kHz) (Fig.1b). The cross matching of the auditory frequency sensitivity to the F_0 of vocalizations between the sexes of *O. tormota* allows frogs of one sex to more reliably perceive calls emitted by the opposite sex, thereby facilitating mate detection in noisy habitat at night. Nevertheless, the mismatch between the female auditory tuning and F_0 of the female calls exists in the frog *O. tormota*, which is different from the findings in other species of frogs [9].

Why don't female *O. tormota* possess the capacity to detect ultrasound? Early studies suggested that the restricted hearing in frogs is largely attributed to the limited high-frequency response of their middle-ear ossicles (ref. 1). The mechanical properties of the middle ear determine the frequency sensitivity of the amphibian auditory system [10], while middle-ear size and ossicular mass in mammals limit the upper frequency of hearing [11]. Adult male and female frogs of *O. tormota* have sexually dimorphic middle ears. Males possess ear canals with a resonant frequency of ~4.3 kHz, which may facilitate high-frequency hearing, and recessed tympana, which shortened and therefore reduced the mass of the middle-ear ossicles. Both low-mass ossicles and thin tympanic membranes facilitate transmission of high-frequency sounds to the inner ear [1]. As contrasted with males, females have relatively large (a diameter of 3.4 ± 0.1 mm, versus 2.6 ± 0.1 mm for males) and thicker (the thinnest part having a thickness of 30-40 µm, versus 3-4 µm in males) tympanic membranes, but no ear canal. The female TM velocity amplitude spectrum showed a distinct peak in sensitivity at frequencies around 5 kHz and much reduced sensitivity above 10 kHz.

Hence, both inertia of the larger and thicker tympanic membranes and heavy-mass ossicles (about 3 times heavier) in females limit the upper frequency of hearing (~16 kHz), approximately a half of the upper frequency limit in males (35 kHz). In comparison, sexual differences in the tympanic frequency responses were also observed in the American bullfrog (*Rana catesbeiana*). The middle ear of adult male bullfrogs showed a distinct peak in sensitivity at very low frequencies around 200 Hz that was entirely lacking in the female middle ear [12, 13]. Males and females did not differ in the frequencies at which they were most sensitive in the green treefrog (*Hyla cinerea*) [14]. It is worth of note that larger tympanic membranes in females of *O. tormota* were responsible for lower auditory thresholds of about 10 dB compared with males, the finding was also observed in two *Hyla* species [15], i.e., females had lower basilar papilla thresholds.

A previous review mentioned that the tonotopic organization of the cochlea plays a crucial role in setting the frequency limits of cochlear sensitivity and hence in determining the bandwidth of hearing in mammals, birds, and reptiles [16]. It remains unknown whether the inner ear (including the hair cells, the basilar papilla and the tectorial membrane) in the concave-eared torrent frogs makes crucial contribution to the determination of the upper-frequency limit of hearing.

METHODS SUMMARY

Animals. Twenty-five female frogs of *Odorrana tormota* were collected at night in Huangshan Hot Springs, China in April and May of 2008 – 2010. Females have very large body size (snout-vent length of 61.9 ± 2.1 mm versus 32.6 ± 1.3 mm in males) and body weight (16.8 ± 2.5 gm versus 3.2 ± 0.3 gm in males).

Behavioural experiments. In a quiet and darkened indoor room, close to the frog' natural habitat, the WAV files of males' calls, including long call, short call, staccato call and their ultrasonic components, were respectively broadcast through a loudspeaker (Fostex FE87E; pass band 0.1–40 kHz) and an ultrasonic loudspeaker (Polaroid; pass band 22–120 kHz) at 90 dB SPL. Using a digital audio recorder (Sound Devices 722) with a 1/4" microphone (40BE, GRAS) and a video camera (SONY HDR-SR7), the evoked vocal responses and phonotaxis of females were recorded under infrared illumination. Phonotaxis analyses were performed as described [2, 5].

Electrophysiology. Sample preparation and electrophysiological recordings (auditory evoked potentials and single-unit activities) from the TS (torus semicircularis) in response to tone bursts (frequency 1-35 kHz) were performed as described [1]. The best excitatory frequency and frequency-threshold curves were determined.

Laser Doppler vibrometry. Using a single-point laser Doppler vibrometer (Polytec OFV-3001, Germany), vibration velocity of the tympanic membrane was measured by a multifunction DAQ (USB-6251, NI) at sample rate of 80 kHz in response to acoustic stimuli, similar to that as described [8].

Detailed descriptions of frog preparation, sound stimuli, phonotaxis experiments, electrophysiology, laser Doppler vibrometry and morphological measurements are provided in the Supplementary Methods.

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Supplementary Information is linked to the online version of the paper at…

Author Contributions

J.X.S. conceived and designed the project. J.X.S. and Z.M.X. performed experiments; Z.L.Y. assisted in part with electrophysiology, S.W. and D.Z.Z assisted with laser Doppler vibrometry. J.X.S. wrote the manuscript.

Competing financial interests

The authors declare no competing financial interests.

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FIGURE LEGENDS

Figure 1|Phonotactic movements and vocal responses in females of concave-eared torrent frog *Odorrana tormota* elicited by playbacks of the male's calls. a, Representative phonotactic responses of gravid females in an arena (150 cm length x 90 cm width), showing long-distance jumps (40-100 cm) with an average localization error of $1.3^{\circ} \pm 1.7^{\circ}$ (mean $\pm$ SD; 60 jumps, 12 females examined) toward the loudspeakers broadcasting the male's long call. Arrows denote the jump's direction. The inset is a picture of a gravid female frog. **b** and **c**, Spectrogram (above) and waveform (below) of an evoked vocal response (EVR) from a gravid female frog (**b**) elicited by the male's long-call stimulus (**c**) with latency of 6.5 s. The EVR consists of a frequency-modulation pip with multiple harmonics having the fundamental frequency (F_0) of 7.9-9.3 kHz (**b**), higher than the F_0 (~6.0 kHz) of the long-call stimulus (**c**).

Figure 2| Averaged auditory-evoked potential (AEP) data from the torus semicircularis (TS) of *O. tormota* to validate extraordinary sex differences in the auditory frequency sensitivity. a, Shown are representative AEP waveforms recorded from the female TS in response to twenty tone bursts presented at frequency range 1-14 kHz, a rate of 1/s and 90 dB SPL. The AEP (*blue*) was recorded at 15 kHz, 103 dB SPL; AEP (*black*) at 18 kHz, 110 dB SPL. **b**, Normalized peak-to-peak amplitudes of the AEPs as a function of tone frequency. Total AEPs $n_f = 95$ (in *red*) in females; $n_m = 61$ (in *blue*) in males. The frequency widths at half maximum are respectively illustrated for males and females in *dashed line*. **c**, Averaged AEP thresholds – frequency

relationships. Total AEPs n_f = 66 (in *red*) in females; n_m = 41 (in *blue*) in males. *Dashed lines* indicate the auditory sensitive frequency bandwidth (at 10 dB above threshold at characteristic frequency, *CF*). Error bars are ± 1 standard error of the mean.

Figure 3| Single-unit best excitatory frequency data from the torus semicircularis (TS) of *O*. *tormota* and vibration velocity amplitude of the tympanic membrane (TM) of females in response to sound. a, Histogram of the best excitatory frequency of the female's 108 single TS units and the male's 110 single TS units, respectively. **b**, Velocity amplitude spectrum of the female's TM, showing a peak around 5 kHz. Measurements at frequency range 1-40 kHz were made from 5 eardrums of 3 females. Error bars are ± 1 standard error of the mean.

METHODS

Animals. Twenty-five female concave-eared torrent frogs of *Odorrana tormota* were collected in Huangshan Hot Springs, China in April and May of 2008 – 2010. Most gravid females were captured during amplexus and then detached (n =22) between 1930 and 2200 hours, but three females were captured while unamplexed.

Behavioural experiments. Females were brought into a quiet and darkened indoor room, ~1 km from the frog's natural habitat for phonotaxis tests. On one side of an arena (150 cm length x 90 cm width), an isolated female frog was placed under a removable glass cover (inside diameter: 8.5 cm), 1 m in front of the loudspeakers. The three basic call-types of males' vocalizations (long call, short call and staccato call)[4] and their filtered ultrasonic components were used as acoustic stimuli. The WAV files of the male's call stimuli were stored on the flash memory of the Play Unit and broadcast through a loudspeaker (Fostex FE87E; pass band 0.1–40 kHz) and an ultrasonic loudspeaker (Polaroid; pass band 22–120 kHz) at 90 dB SPL and a rate of one call per 15 s. Using a digital audio recorder (Sound Devices 722, USA; 24 bit, 192 kHz sample rate) with a 1/4" precision microphone (40BE, GRAS Denmark; 4 Hz – 100 kHz) mounted on a tripod and placed 10 cm from the loudspeakers and a video camera (SONY HDR-SR7), the evoked vocal responses and phonotaxis of females were recorded under infrared illumination. The trajectories for each female frog were obtained from the video recordings. The jump distance and azimuthal angle were measured. Phonotaxis analyses were performed as described[5].

Electrophysiology. Seventeen females and thirty males were carried into the Institute

of Biophysics in Beijing, China for electrophysiological study. Frogs were deeply anaesthetized by immersion in a 0.3% solution of tricaine methanesulfonate (MS222) and wrapped in cotton gauze. Incisions were made in the skin on the dorsal surface of the head, and a small hole was made in the skull above the torus semicircularis (TS). After surgery, animals were placed on a rubber platform inside a sound-proof and anechoic room, and immobilized during the recording session with periodic addition of 0.1% MS222. Tone bursts (50-ms duration, 5-ms rise and fall times, presented at a rate of 1/s) were generated by an RP2.1 Enhanced Real-time Processor [Tucker Davis Technologies (TDT) System 3], and broadcasted from a loudspeaker (FE87E, Fostex Japan) positioned 50 cm from the frog's contralateral eardrum. The sound pressure levels of the stimulation system were measured with a condenser microphone (Brüel and Kjaer 4135) and a sound level meter (Brüel and Kjaer 2610). Glass microelectrodes (impedance 1-10 MΩ) were used to record auditory evoked potentials (AEPs; averaged over 20 trials) and single-unit activities from the TS in response to tone bursts at frequency range 1-35 kHz. Neural signals were amplified (RA4PA Preamp and RA16 Medusa Base, TDT), monitored visually and extracted using BrainWare software and stored on a hard drive and analyzed off-line. The best excitatory frequency and frequency-threshold curves were determined for single TS units.

Laser Doppler vibrometry. Using a single-point Doppler laser vibrometer (Polytec OFV-3001, Germany), the vibration velocity of the tympanic membrane (TM) of frogs was measured in response to acoustic stimuli at an angle normal to the TM. The

frog was anesthetized by immersion in a bath of 0.3% MS-222 for 5 min. A single retroreflective hemispheric glass bead (diameter 30-50 μm; 3M Scotchlite) was carefully placed in the center of the TM with fine-tipped tweezers to increase its reflectivity and maximize measurement precision. A custom-made foam base (4 cm length x 3 cm height x 2.5 cm width) where the female frog rested from the vent to the pectoral girdle was used to support the frog during vibrometry. As acoustic stimuli, tone bursts (5-ms rise-fall time, 50 ms duration, frequency 1-40 kHz, a rate of 1/s) were generated by a function generator (DG3061A, Rigol China) and were broadcast from a loudspeaker (1-40 kHz; FE87E, Fostex) positioned at 25 cm from the TM. Vibration velocity amplitude of the TM was recorded by a Multifunction Data Acquisition DAQ (USB-6251, NI USA) at sample rate of 80 kHz.

Morphology. The male and female frogs of *O. tormota* show evident sexual dimorphism (mean ± SD): snout-to-vent length 32.6 ± 1.3 mm in males ($n = 8$) versus 61.9 ± 2.1 mm in females ($n = 5$); head width 9.7 ± 0.3 mm in males versus 14.3 ± 0.3 mm in females; body weight 3.2 ± 0.3 g in males versus 16.8 ± 2.5 g in females. The morphology of eardrum was measured based on horizontal sections stained with haematoxylin-eosin (50 μm in thickness). The eardrum diameter 2.6 ± 0.1 mm in males versus 3.4 ± 0.1 mm in females; thickness of eardrum at the rim 3-4 μm in males versus 30-40 μm in females. The middle-ear ossicles were removed from anaesthetized frogs and weighed by an electronic balance (Sartorius BP211D; Readability 0.01 mg): 0.1 mg in males versus 0.23 mg in females.
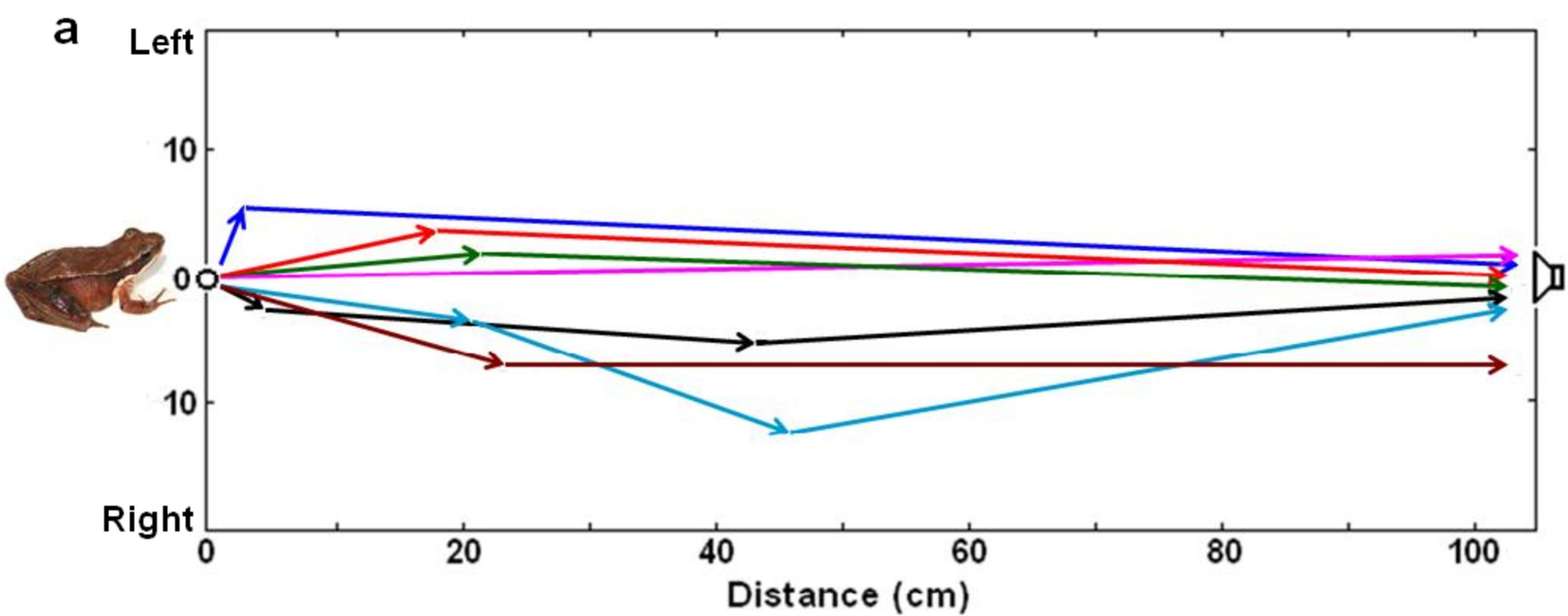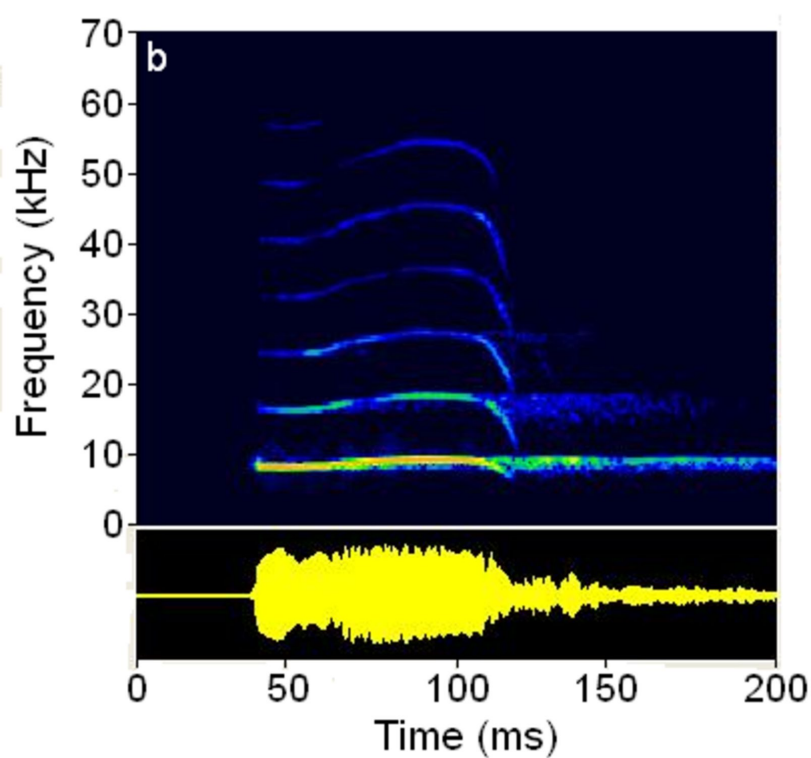
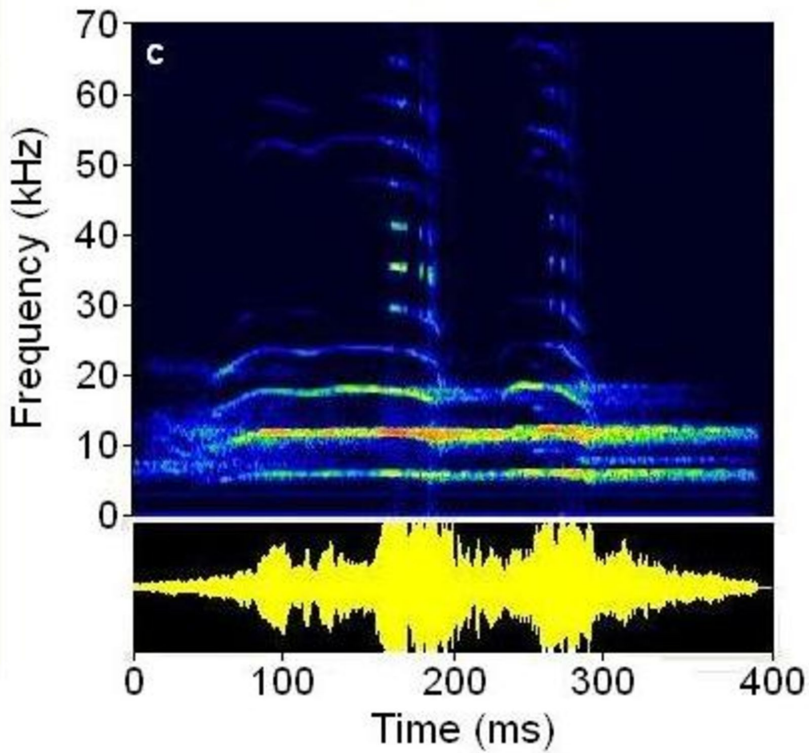# Supplementary Information

SUPPLEMENTARY MOVIE LEGENDS

Supplementary Movie SM-1a, SM-1b| Phonotactic behaviour in female *Odorrana tormota* evoked by the male's long call. SM-1a, Heard the male's long call from the loudspeakers, a gravid female immediately turned toward sound source, then jumped about 90 cm distance and stayed beside the speakers. **SM-1b**, Elicited by the male's long call, another gravid female directly jumped about 90 cm toward the speakers and stayed in front of the speakers. Windows Media Video (**SM-1a**, 201 KB; **SM-1b**, 568 KB).

Supplementary Movie SM-2| Phonotactic behaviour in female *Odorrana tormota* evoked by the male's short call. Heard male's short call, the gravid female prepared for action, then jumped twice (each jump about 30 cm) toward the loudspeakers, and finally hit against the speakers. Windows Media Video (342 KB).
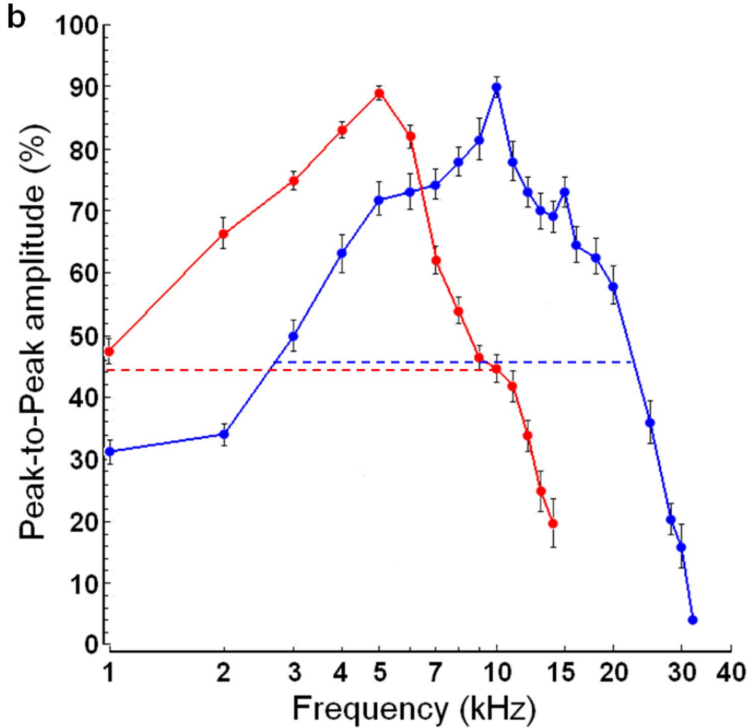
Supplementary Movie SM-3a, SM-3b| Female phonotactic behaviour and antiphonal vocalizations evoked by the male's long call. SM-3a, A female frog firstly turned, then jumped (~30 cm) and crept (~20 cm) toward the loudspeakers, stayed in the way and thrice emitted antiphonal calls with the fundamental frequency (F_0, ~8.1 kHz) higher than the F_0 of the male's long call (~ 6.0 kHz). Windows Media Video (1,092 KB). **SM-3b**, Another gravid female frog continuously crept about 65 cm toward the loudspeakers and then emitted antiphonal short high-frequency pips (total 8 calls) around the speakers. Windows Media Video (483 KB).
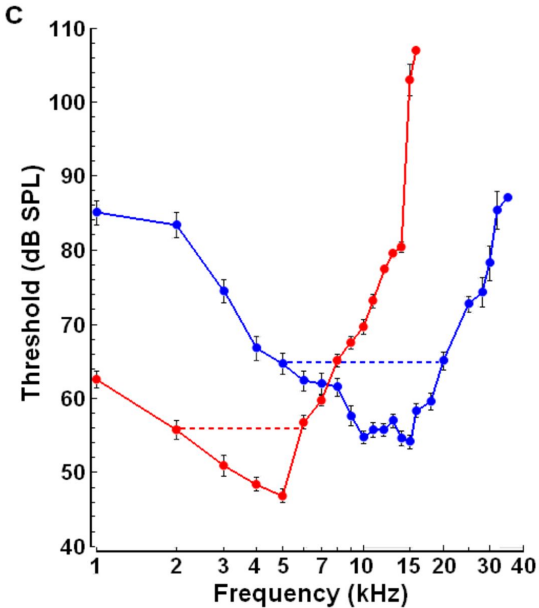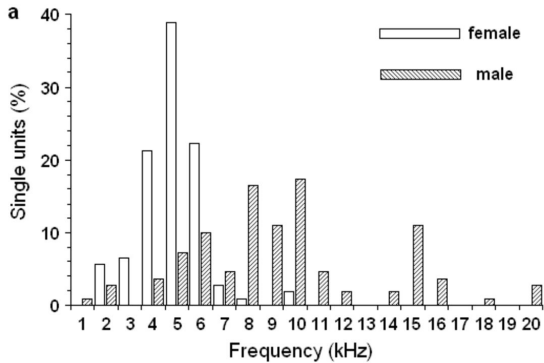
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